PROLIFERATIVE RESPONSES OF NORMAL RAT VENTRAL PROSTATE 'IN VITRO'

Lynn-Joy Buchanan

A Thesis Submitted for the Degree of PhD
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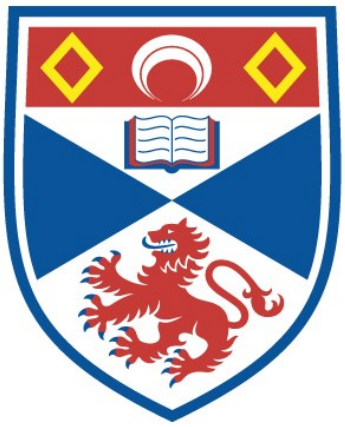

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ABSTRACT

Proliferative responses of normal rat ventral prostate *in vitro* were investigated using the incorporation of 5-[^{125}I]-iodo-2'-deoxyuridine (^{125}I-UdR) to monitor DNA synthesis, with the aim of establishing a model system for evaluating the direct effects of hormones and chemotherapeutic agents on prostatic growth. In androgen-free, chemically-defined organ culture, the proliferative activity of young adult rat (4 to 6 months old) ventral prostate declined with time and the tissue underwent retrogressive changes resembling post-castration atrophy *in vivo*. Treatment with testosterone (4×10^{-12} to 4×10^{-5}M) exhibited a dose-dependent response, with concentrations ranging from 4×10^{-9} to 4×10^{-6}M preventing the retrogressive changes associated with androgen deprivation and eliciting maximal increases (approximately 3-fold) in ^{125}I-UdR uptake. Higher testosterone concentrations (2.5×10^{-5} to 4×10^{-5}M) exerted a non-specific cytotoxic effect, resulting in marked suppression of ^{125}I-UdR uptake. Following stimulation with an optimal concentration of testosterone (4×10^{-7}M), ^{125}I-UdR incorporation reached peak activity on day 4 of the culture period and rapidly declined thereafter, despite the continued presence of testosterone. Effects of variations in organ culture media and methodology were also examined to further establish optimal conditions for investigations of the proliferative response to testosterone stimulation.

The established method of quantitative organ culture was then used to compare the proliferative effect of testosterone with that of its major metabolites, 5α-dihydrotestosterone, androstenedione, androstanedione and 5α-androstane-3β, 17β-diol.

PROLIFERATIVE RESPONSES OF NORMAL RAT VENTRAL PROSTATE IN VITRO

by

Lynn-Joy Buchanan

A thesis presented for the degree of Doctor of Philosophy

at the University of St. Andrews

Department of Anatomy and Experimental Pathology

University of St. Andrews

April 1983

DECLARATION

I declare that this thesis is a record of my own work and that it has not been submitted for any other degree.

Lynn-Joy Buchanan

St. Andrews, April 1983

SUPERVISOR'S CERTIFICATE

I certify that Lynn-Joy Buchanan has spent 12 terms of research under my supervision, that she has fulfilled the conditions of Ordinance General No. 12 and Resolution of the University Court 1967 No. 1, and that she is qualified to submit this thesis for the degree of Doctor of Philosophy.

Dr. A.C. Riches

St. Andrews, April 1983

Dedicated with affection and gratitude to

my mother

and in memory of

my father

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CONTENTS

ABBREVIATIONS AND SYMBOLS

A-3,17-dione	androstanedione
A-3β,17β-diol	androstane-3β,17β-diol
Δ^4-A-3,17-dione	androstenedione
BPH	benign prostatic hyperplasia
°C	degrees centrigrade
CBG	corticosteroid binding globulin
cc	cubic centimetre
Ci	curie
CO_2	carbon dioxide
CPM	counts per minute
DHT	5α-dihydrotestosterone
5β-DHT	5β-dihydrotestosterone
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
E_2-17β	oestradiol-17β
FCS	foetal calf serum
g	gram
h	hour
HEPES	N-2-hydroxyethyl-piperazine-N'-2-ethane sulphonic acid
IU	international unit

^{125}I-UdR	5-^{125}Iodo-2'-deoxyuridine
L	laevo
LH	lutenizing hormone
LH-RF	lutenizing hormone-releasing factor
LS 275	estramustine
LS 299	estramustine phosphate; Estracyt^{R} (free acid)
LS 299Z	estramustine phosphate sodium; Estracyt^{R} (disodium salt)
M	molar
mg	milligram
ml	millilitre
mm	millimetre
mM	millimolar
mm^3	cubic millimetre
mmole	millimolar
MW	molecular weight
μCi	microcurie
μg	microgram
μl	microlitre
μm	micrometre
n	number of replicate experiments
N	normal
NaOH	sodium hydroxide
NPH	nodular prostatic hyperplasia
PAS	periodic-acid Schiff

RNA	ribonucleic acid
rpm	revolutions per minute
SBP	sex steroid binding plasma protein
s.e.m.	standard error of the mean
SHBG	sex steroid hormone binding globulin
T	testosterone
TCA	trichloroacetic acid
^{3}H-TdR	[methyl-^{3}H]-thymidine
TEBG	testosterone oestradiol binding globulin
TUR	transurethral resection
>	greater than
≥	greater than or equal to
<	less than

TRIVIAL NAMES AND SYSTEMATIC EQUIVALENTS

Androstane-3α,17β-diol	5α-androstane-3α,17β-diol
Androstane-3β,17β-diol	5α-androstane-3β,17β-diol
Androstanedione	5α-androstane-3,17-dione
Androstenedione	androst-4-ene-3,17-dione
Androsterone	3α-hydroxy-5α-androstan-17-one
Cyproterone acetate	1,2α-methylene-6-chloro-$\Delta^{4,6}$-pregnadiene-17α-ol-3,20-dione-17α-acetate
Diethylstilboestrol	trans-3,3-bis(p-hydroxyphenyl) hex-3-ene
5α-dihydrotestosterone	17β-hydroxy-5α-androstan-3-one
5β-dihydrotestosterone	etiocholan-17β-ol-3-one
Estramustine	estra-1,3,5(10)-triene-3,17β-diol,3-/N,N-bis-(2-chloroethyl carbamate
Estramustine phosphate	estra-1,3,5(10)-triene-3,17β-diol,3-/N,N-bis-(2-chloroethyl carbamate/-17-dihydrogen phosphate
Estramustine phosphate sodium	estra-1,3,5(10)-triene-3,17β-diol,3-/N,N-bis-(2-chloroethyl carbamate/-17-disodium phosphate
Isoandrosterone (Epiandrosterone)	3β-hydroxy-5αandrostan-17-one
Oestradiol-17β	estra-1,3,5(10)-triene-3,17β-diol
Testosterone	17β-hydroxy-androst-4-en-3-one

CHAPTER 1

INTRODUCTION

1.1 INTRODUCTION TO THE MALE ACCESSORY SEX GLANDS

A unique characteristic of the reproductive system in male mammals is the development of discrete accessory sex glands. These glands are androgen-dependent organs which develop in association with specific regions of the male urogenital tract and function primarily in the production of seminal fluid (Mann, 1954, 1964, 1974; Mann and Lutwack-Mann, 1981). The male accessory sex glands include the seminal vesicles, the ampullary glands, the bulbourethral glands and the prostate gland, but considerable species-specific variation exists with regard to which of these glands are present (Price and Williams-Ashman, 1961). All male mammals do, however, possess at least one accessory sex gland and only the prostate has been universally found in all orders of mammals (Price, 1963).

1.1.1 Pathology of the Human Prostate Gland

While proliferative lesions and neoplasms are uncommon in most of the accessory reproductive glands, much biomedical interest and research has been focused on the prostate as a result of the frequent occurrence, and clinical importance, of benign prostatic hyperplasia (BPH) and prostatic cancer in aging men. BPH is the most common urologic disease in middle-aged and elderly men (Rotkin, 1975; Scott and Coffey, 1975; Elbadawi, 1979b, 1980) and the most frequent cause of infravesical obstruction in the human male (Elbadawi, 1979b, 1980), while prostatic cancer is the most common malignancy of the male urogenital tract (Chisholm, 1981) and a leading

cause of cancer mortality (Winkelstein and Ernster, 1979; Chisholm, 1981; Ablin, 1981; Greenwald, 1982; Baba, 1982). The most common form of BPH, known as nodular prostatic hyperplasia (NPH), consists of multicentric nodules of stromal, glandular or stromoglandular tissue (Franks, 1975; Elbadawi, 1979b, 1980) which characteristically arise in the periurethral region of the prostate (Scott, 1963; McNeal, 1972, 1978; Franks, 1975; Elbadawi, 1979b, 1980; Blacklock, 1981). While cancer of the prostate occurs in men of the same age group as BPH, it is characterized by the following: (1) prostatic cancer may be multifocal in origin (Elbadawi, 1979a; Glenn, 1981) but the most frequent site of development is in the periphery of the gland, particularly on the posterior aspect (McNeal, 1968, 1969, 1975b, 1980b; Franks, 1973; Grayhack and Wendel, 1974; Blacklock, 1981), (2) histologically, most prostatic tumours (> 95%) are adenocarcinomas which commonly exhibit varying degrees of differentiation (Grayhack and Wendel, 1974; Gleason, 1977; Gaeta and Gardner, 1979; Elbadawi, 1979b; Bruce and Mahan, 1980; Tannenbaum and Romas, 1981; Dhom and Hohbach, 1982), (3) prostatic tumours spread by direct local invasion and via lymphogenous and haematogenous dissemination, with the latter frequently giving rise to osseous metastases, particularly in the pelvic bones and lumbosacral vertebrae (Elbadawi, 1979b; Bruce and Mahan, 1980; Glenn, 1981) and (4) 70 to 90% of patients with bone metastases secondary to prostatic cancer exhibit elevated serum prostatic acid phosphatase levels (Gutman and Gutman, 1938b; Robinson et al., 1939; Huggins and Hodges, 1941; Sodeman and Batsakis, 1977; Wajsman and Chu, 1979; Bruce and Mahan, 1980; Rose et al., 1981; Prellwitz and Ehrenthal, 1982).

Despite extensive research, BPH and prostatic cancer are proliferative diseases of unknown aetiology. Since both conditions are common and share a progressively increasing incidence with age, BPH has been considered as a possible precursor of prostatic cancer (Greenwald *et al.*, 1974; Armenian *et al.*, 1974). However, the relationship, if any, between these two diseases remains unresolved (Murphy, 1977; Winkelstein and Ernster, 1979; Rotkin, 1975; 1980; Baba, 1982). Extrinsic (chemical, physical and viral) and intrinsic (genetic and hormonal) factors have also been considered in the aetiology of prostatic cancer, but none have been conclusively proven to be direct causative agents (Winkelstein and Ernster, 1979). Although the classic work of Huggins and colleagues (Huggins and Clark, 1940; Huggins and Hodges, 1941; Huggins *et al.*, 1941) clearly established the androgen-dependence of some prostatic tumours by demonstrating regression of both primary and secondary tumours following castration and/or oestrogen therapy, the involvement of hormonal factors in the aetiology of this disease remains poorly understood and, as yet, there is no direct evidence that hormones can initiate cancer of the prostate (Harper *et al.*, 1981). At present, the single undisputed factor in the aetiology of prostatic cancer is that it only develops spontaneously in aged men who were not castrated prepubertally.

1.1.2 Clinical Management of Prostatic Cancer

While cancer of the prostate is potentially curable in its initial stages, the detection of early, surgically removable tumours is uncommon as most patients (> 80%) are in advanced stages of the disease when it is first diagnosed

(Murphy, 1977; Wajsman and Chu, 1979; Bruce and Mahan, 1980; Altwein, 1982). Moreover, the age and general health of the patient are often a contraindication to radical surgery.

Since Huggins and Hodges (1941) first demonstrated the effectiveness of hormonal manipulation in the treatment of prostatic cancer, endocrine therapy has remained the major therapeutic modality for metastatic carcinoma of the prostate. As the rationale for this treatment is based on the ablation of androgenic stimuli, endocrine manipulations used in the management of prostatic cancer have included bilateral orchiectomy, oestrogen therapy, adrenalectomy or hypophysectomy or a combination of these modalities (Scott, 1973; Chisholm and O'Donoghue, 1975; Menon and Walsh, 1979; Murphy, 1979; Scott et al., 1980; Yagoda, 1981; Altwein, 1982). While treatment with the synthetic oestrogen, diethylstilboestrol (DES), constitutes the conventional form of endocrine therapy, clinical evidence has suggested that it is associated with serious cardiovascular side-effects which may negate the therapeutic value of the treatment (VACURG, 1967). Consequently, there has been an increased tendency toward the use of bilateral orchiectomy as a primary form of endocrine therapy (Barnes, 1981).

Uncertainty about the consequences of oestrogen treatment has also stimulated interest in the use of antiandrogens as an alternative form of therapy for the management of prostatic cancer. In contrast to oestrogens, which suppress testicular androgen biosynthesis indirectly via the gonadal-pituitary-hypothalamic axis, antiandrogens exert their effect primarily by direct competition with androgens at the target cell level (Dorfman, 1967, 1970; Steinbeck et al., 1971; Neumann and Steinbeck, 1974;

Neumann and Schenck, 1976; Neri, 1976; Menon and Walsh, 1979; Mittelman, 1979; Tveter et al., 1980; Neumann et al., 1976; 1980). Among synthetic, steroidal antiandrogens, the hydroxyprogesterone derivative known as cyproterone acetate, has been most extensively investigated and shown to antagonize androgen-mediated responses in the prostate, including RNA and DNA synthesis (Fang et al., 1969; Jäger et al., 1969; Geller et al., 1969; Belham and Neal, 1971; Walsh and Korenman, 1971; Carter et al., 1972; Sufrin and Coffey, 1973, 1975; Liao et al., 1974; Coffey, 1974; Mainwaring, 1977; Tveter et al., 1980). Numerous experimental studies have further shown that treatment of normal animals with cyproterone acetate causes marked involution of the prostate, similar to that following surgical castration (Neumann et al., 1967, 1976, 1980, 1982; Neumann, 1971, 1977; Steinbeck et al., 1971; Neumann and Steinbeck, 1974; Neumann and Schenck, 1976; Tveter et al., 1975, 1980). Clinical evidence also suggests that cyproterone acetate is effective in the treatment of prostatic cancer and produces fewer side-effects than conventional oestrogen therapy (Neumann, 1971, 1977; Wein and Murphy, 1973; Neumann et al., 1976, 1982; Isurugi et al., 1980; Jacobi et al., 1982), although Tveter et al. (1978, 1980) have reported that the incidence of cardiovascular side-effects is comparable to that with oestrogen therapy. At present, clinical experience with cyproterone acetate has been largely limited to patients with metastatic carcinoma of the prostate refractory to hormonal therapy, thus the true value of this drug as an alternative to conventional oestrogen therapy remains to be established (Neumann et al., 1980, 1982; Smith, 1981; Jacobi et al., 1982; Gupta, 1982; Pavone-Macaluso, 1982).

Despite the continued use of endocrine therapy in the treatment of prostatic cancer, it does not constitute a cure for this disease but, rather, serves as a temporary palliative measure. While the majority of patients initially respond to endocrine treatment, most eventually relapse to a hormone-resistant state and at least 15 to 20% fail to respond at all (Nilsson and Jönsson, 1977; Menon and Walsh, 1979; Barnes, 1981). Hence, the response to this treatment is often variable and, as yet, there is no method of predicting which patients will respond to endocrine manipulation (Karr and Sandberg, 1979; Ekman et al., 1979; Wagner, 1980; Pfitzenmaier et al., 1980; Concolino et al., 1980; Ekman, 1980; Mobbs et al., 1980; de Voogt and Dingjan, 1980; Rennie and Bruchovsky, 1980; Brandes et al., 1981; Harper et al., 1981; de Voogt, 1982).

The lack of hormonal response in some patients, along with the eventual relapse which occurs in patients initially responding to hormonal therapy, has stimulated a search for alternative chemotherapeutic approaches to the treatment of prostatic cancer. Until recently, chemotherapy has received little attention as a therapeutic modality for prostatic cancer primarily because of the relative success in managing this disease with endocrine therapy (Nilsson and Müntzing, 1980; Murphy and Slack, 1981). Adverse side-effects often associated with conventional cytotoxic agents have also contributed to the reluctant attitude toward chemotherapy (Murphy, 1979; Nilsson and Müntzing, 1980). However, the introduction of hormone-cytotoxic agents specifically designed for the treatment of hormonally-linked tumours has stimulated renewed interest in the use of chemotherapy for prostatic cancer (Murphy, 1979; Nilsson and Müntzing, 1980;

Murphy and Slack, 1981; Pavone-Macaluso, 1982). The rationale for hormone-cytotoxic therapy is based on the concept that linking a cytotoxic agent to a steroid carrier-hormone may enhance the tissue specificity of the compound and perhaps reduce the systemic side-effects caused by either substance alone. Theoretically, the hormonal moiety imparts tissue specificity by interacting with steroid hormone receptor proteins in the target organ, thereby resulting in an accumulation of the alkylating agent within the tissue. Subsequent hydrolysis of the drug complex releases the cytotoxic agent in an active form, and both the hormonal and cytotoxic moieties may then exert their effects independently (Mittelman, 1979; Nilsson and Müntzing, 1980; Pavone-Macaluso, 1982). Among such compounds developed for the treatment of prostatic cancer, the nor-nitrogen mustard derivative of oestradiol-17β, known as estramustine phosphate or Estracyt^{R}, has been most extensively investigated. Both in the rat and man, estramustine phosphate is rapidly dephosphorylated to estramustine (Høisaeter, 1976a, 1976b, 1977; Edsmyr et al., 1982). Experimental studies have further shown that following the administration of ^{3}H-estramustine phosphate to rats, radioactivity is preferentially localized in the prostate and is primarily associated with the metabolite, estramustine (Plym-Forshell and Nilsson, 1974; Forsberg and Høisaeter, 1975; Høisaeter, 1976a, 1977), indicating that the intact steroid-carbamate complex is incorporated into the prostate (Edsmyr et al., 1982). In the rat, treatment with estramustine phosphate reportedly causes reductions in the weight of the ventral prostate (Kirdani et al., 1974; Yamanaka et al., 1977), decreased 5α-reductase activity (Kirdani et al., 1974; Forsberg and Høisaeter, 1975;

Høisaeter, 1975a, 1977) and inhibition of androgen-induced DNA synthesis (Forsberg and Høisaeter, 1975; Høisaeter, 1975b, 1976b, 1977), but its precise mode of action is unknown (Forsberg and Høisaeter, 1975; Høisaeter, 1975a, 1975b, 1976b, 1977; Nilsson and Müntzing, 1980; Edsmyr et al., 1982). Clinically, selective accumulation of radioactivity has also been detected in the human prostate gland following the administration of ^{131}I-estramustine phosphate (Szendröi et al., 1973). Clinical evidence has further indicated that Estracyt is often effective in the treatment of oestrogen-relapsed prostatic carcinoma (Jönsson and Högberg, 1971; Nilsson and Müntzing, 1972, 1980; Müntzing et al., 1974; Murphy, 1975; Jönsson et al., 1975; Mittelman et al., 1975, 1977; Nilsson and Jönsson, 1977; Yamanaka et al., 1981b; Edsmyr et al., 1982), suggesting that its mode of action differs from that of oestrogens. Several studies have also found that Estracyt produces few side-effects (Jönsson and Högberg, 1971; Müntzing et al., 1974; Jönsson et al., 1975; Murphy, 1975; Mittelman et al., 1975, 1977; Nilsson and Jönsson, 1977), although Yamanaka et al. (1981b) and Edsmyr et al. (1982) have reported that the untoward effects of Estracyt therapy are comparable to those associated with oestrogen treatment. As with cyproterone acetate, the true value of Estracyt as an alternative to primary oestrogen therapy remains to be established since clinical experience with this drug has generally been restricted to patients with hormone-resistant prostatic carcinoma (Nilsson and Jönsson, 1977; Nilsson and Müntzing, 1980; Smith, 1981; Edsmyr et al., 1982; Pavone-Macaluso, 1982).

While insight into the molecular endocrinology of the prostate has had a profound influence on the development

of drugs for the treatment of prostatic cancer (Mittelman, 1979), the sobering fact remains that the five year survival rate for this disease is only 57% (Ablin, 1981). Moreover, little is known of the factors involved in the regulation of prostatic growth.

1.1.3 Animal Models in Prostate Research

Research into the abnormal growth of the prostate, and its control, has been impeded by a lack of suitable animal models. With the exception of senescent dogs, BPH rarely occurs spontaneously in subhuman animals. Although the natural history of canine BPH is remarkably similar to that in the human (Walsh, 1975; Sandberg et al., 1980), the two conditions differ in their histological characteristics and endocrine responses (Ofner, 1968; Walsh, 1975; Brendler, 1975; Scott and Coffey, 1975; Neumann et al., 1975; Sandberg et al., 1980). Despite these differences, however, canine BPH is considered to be the most promising animal model currently available for studying the pathogenesis of BPH (Walsh, 1975; Sandberg et al., 1980; Chevalier et al., 1981).

In contrast to the frequent occurrence of BPH in senescent dogs, prostatic cancer seldom develops spontaneously in this species. Although malignant tumours have been found in aged dogs (Brendler, 1963; Ofner, 1968; Leav and Ling, 1968; Sandberg et al., 1980), the incidence is probably less than 1%, thus precluding its use as a model for human prostatic cancer (Brendler, 1963; Neumann et al., 1975; Coffey et al., 1979; Issacs and Coffey, 1979, 1981).

BPH and prostatic cancer have also been reported to occur spontaneously in aged, non-human primates (Lewis and Kaack, 1980; Lewis et al., 1981) but these animals have not

yet been adequately studied to determine the true spontaneous incidence of either disease (Coffey and Issacs, 1980; Lewis et al., 1981). The long lifespan of these animals may, however, become a limiting factor in their use as an experimental model for abnormal prostatic growth.

Spontaneous BPH is not known to occur in rats (Price and Williams-Ashman, 1961; Walsh, 1975) and until recently, prostatic cancer was considered to be extremely rare in this species (Price and Williams-Ashman, 1961; Brendler, 1963; Dunning, 1963; Franks, 1967; Franks and Maldague, 1976; Burek, 1981). However, in aged (31 to 39 months old), germ-free Lobund-Wistar rats the spontaneous incidence of prostatic adenocarcinomas has been found to be 13%, and in animals beyond 35 months of age the incidence is 35% (Pollard, 1973; Pollard and Luckert, 1975). Similarly, Shain et al. (1975, 1977, 1979) have reported that spontaneous prostatic adenocarcinomas occur in senescent (34 to 37 months old), conventionally-reared AXC rats with a frequency of 17% and in animals ranging from 30 to 46 months of age the incidence is 57%. Shain et al. (1977, 1979) also found that in testosterone-treated, aged AXC rats the incidence of prostatic tumours increased to 70%. Similarly, Noble (1977a, 1977b) reported that the low (0.48%) spontaneous incidence of prostatic adenocarcinomas in 13 to 14 month old Nb rats increased significantly (18.38%) following prolonged (9 to 18 months) treatment with testosterone and estrone implants. Hence, the general assumption that prostatic cancer rarely occurs in rats is no longer justified. Moreover, several important transplantable rat prostatic tumour models, notably the Dunning R-3327 series (Dunning, 1963; Lubaroff et al., 1980),

the Pollard tumours (Pollard and Luckert, 1975; Pollard, 1980), the AXC tumours (Shain et al., 1977, 1979) and the Nb tumours (Noble, 1977a, 1977b; Drago et al., 1980a) have been established by successful propagation of these spontaneous and hormone-induced adenocarcinomas. Detailed reviews of the known properties of these rat prostatic tumour models have been presented recently (Müntzing et al., 1977; Smolev et al., 1977; Issacs and Coffey, 1979, 1981; Lubaroff et al., 1980; Drago et al., 1979, 1980a, 1980b; Drago and Goldman, 1980; Pollard, 1980; Bogden, 1980; Issacs et al., 1980; Schröder, 1982) and indicate that these tumours exhibit a wide range of histology, growth rates, metastasizing potential and sensitivity to hormones and chemotherapeutic agents. Although no single rat prostatic tumour appears to encompass the diversity of human prostatic cancer, these tumours represent the most promising animal models presently available for investigations into the aetiology, pathogenesis and treatment of prostatic cancer.

Apart from hormone-induced tumours, research efforts have also focused on the development of carcinogen-induced prostatic adenocarcinomas in vivo (Dunning, 1963; Brendler, 1963; Fraley and Paulson, 1974; Neumann et al., 1975; Pour, 1981) and in vitro (Lasnitzki, 1951, 1954, 1964, 1974; Fraley and Paulson, 1974; Neumann et al., 1975; Norris, 1980). These efforts have, however, failed to produce an experimental tumour which is a suitable analogue for human prostatic cancer.

While several promising animal models for BPH and prostatic cancer are currently available, it must be emphasised that no single animal prostate, either normal or neoplastic, has emerged as an ideal surrogate for the human prostate gland. Factors contributing to this problem include marked

species-specific differences in the morphology, biochemistry and pathology of the prostate (Price and Williams-Ashman, 1961; Price, 1963; Van Camp, 1969; Mann and Lutwack-Mann, 1981), as well as the general lack of agreement regarding the regional morphology of the normal human prostate (Scott, 1963; McNeal, 1968, 1972, 1980a; Tisell and Salander, 1975; Elbadawi, 1979a; Aumüller, 1979). Furthermore, cancer of the prostate in man is, itself, a highly variable and multifaceted disease in which no two tumours are identical and often a single tumour exhibits marked heterogeneity. Thus the quest for a single, ideal animal model for human prostatic cancer seems to be an unrealistic goal as it appears that several models may be necessary to adequately represent the diversity of the natural disease in man (Coffey et al., 1979; Issacs and Coffey, 1979, 1981; Merchant, 1980; Schröder, 1982). For similar reasons, Sandberg and colleagues (Sandberg, 1975; Sandberg et al., 1977; Sandberg and Kadohama, 1980) have stressed the need for multiple in vivo and in vitro model systems to assess the effects of drugs which may be of potential value in the clinical management of prostatic cancer. Time, cost and ethical considerations further necessitate the use of experimental models to develop and evaluate new modalities of therapy.

While the merit of animal studies in prostate research has long been recognized as a result of their great contribution to the basic knowledge of human prostate biology and their influence on the development of therapeutic concepts for the management of prostatic cancer, the extrapolation of results from animal studies to the human must always be cautiously guarded (Merchant, 1979; Coffey and Issacs, 1980). Handelsman (1977) has further stressed that any model system

is limited in the information that it can provide, therefore it is essential that animal models used in prostate research be well-characterized and demonstrate unique properties which correspond with the human prostate gland (Sandberg et al., 1977, 1980; Coffey et al., 1979; Coffey and Issacs, 1980; Merchant, 1980; Schröder, 1982). Clearly, differences between the human prostate and the proposed animal model must also be taken into consideration if experimental results are to provide a better understanding of prostatic diseases in man.

1.1.4 Developmental and Comparative Anatomy of the Human and Rat Prostate Glands

Among experimental animals most frequently used in prostate research has been the normal laboratory rat (Rattus norvegicus albinus). Although the rat and human prostate glands exhibit distinct morphological and biochemical differences, both organs follow a similar pattern of development (Price, 1963; Narbaitz, 1974).

Embryogenesis of the human prostate was extensively studied by Lowsley (1912) and has recently been the subject of several reviews (Narbaitz, 1974; McNeal, 1975a; Aumüller, 1979; Cunha and Lung, 1979a, Jirásek, 1980). At approximately the twelfth week of gestation, primordia of the human prostate appear as short, solid cords of epithelial cells growing out from the cephalic portion of the urogenital sinus into the surrounding mesoderm. Development of the prostatic primordia appears to be dependent on the production of androgens by the foetal testis (Kellokumpu-Lechtinen and Santti, 1979) which occurs during the tenth to twelfth week of gestation (Price et al., 1975; Faiman et al., 1981). In

a thirteen week human foetus, Lowsley (1912) identified the following five groups of epithelial cords arising from the prostatic urethra: (1) a middle, or median, group originating from the posterior wall of the urethra between the bladder neck and the openings of the ejaculatory ducts and the prostatic utricle, (2) and (3) right and left lateral groups arising from the prostatic furrows and lateral walls of the urethra on either side of the verumontanum, (4) a posterior, or dorsal, group arising from the posterior wall of the urethra, caudad to the openings of the ejaculatory ducts, and (5) a small anterior group arises from the ventral wall of the urethra craniad to the prostatic utricle, but atrophies almost completely after the sixteenth week in utero. According to Lowsley's (1912) definition, the glands arising from these foci give rise to five separate lobes of the foetal prostate, namely: the middle lobe, the right and left lateral lobes, the posterior lobe and the anterior lobe, respectively. However, as the prostatic cords grow longer and branch the boundaries separating the lobes become obscured, and the ejaculatory ducts and prostatic utricle become incorporated into the substance of the prostate gland (Narbaitz, 1974; Cunha and Lung, 1979a). Between the thirteenth and sixteenth weeks of gestation, lumenization of the epithelial cords begins and proceeds from the entrance of the glands into the urethra toward the periphery. At about the same stage of development, muscular differentiation and collagen fibrilogenesis occur in the surrounding mesenchyme (Cunha and Lung, 1979a).

At birth, the five lobes of the foetal prostate, as described by Lowsley (1912), are no longer evident (McNeal, 1975a; Franks, 1975). Differentiation of the epithelial

cords in the neonatal prostate is incomplete as only some glands have developed lumina lined with single or stratified cuboidal epithelium, while the terminal portions of others remain solid. Evidence of squamous metaplasia in the foetal ($\geq$ 22 weeks) and neonatal prostate has been attributed to the influence of maternal oestrogens (Zondek and Zondek, 1975, 1980). Regression of these metaplastic changes occurs gradually after birth.

Postnatally, differentiation and growth of the human prostate continues slowly until puberty. At this time prostatic growth accelerates rapidly in response to elevated androgen levels and the prostate gland achieves morphological and functional maturity.

The normal anatomy of the adult human prostate has been described in detail by Gray (1980) and discussed in reviews by Narbaitz (1974), Aumüller (1979) and Spring-Mills and Hafez (1979, 1980). The normal adult prostate is a compact fibromuscular and glandular organ, which is almost conical in shape, and lies obliquely in the pelvic cavity between the symphysis pubis and the rectum, surrounding the urethra at the neck of the bladder. In a healthy male adult, the prostate is the largest of the accessory sex organs, measuring 3 to 4cm transversely at the base, approximately 2cm antero-posteriorly and 3cm vertically; and weighs about 20g.

The base of the prostate is directly contiguous with the inferior surface of the bladder and is pierced at its anterior border by the urethra. The prostatic portion of the urethra passes vertically through the prostate and emerges slightly anterosuperior to the apex of the gland, which lies at the level of the urogenital diaphragm.

The posterior aspect of the prostate is a triangular,

flattened surface which is often marked with a shallow, longitudinal furrow. The two ejaculatory ducts pierce this surface near its superior border, pass obliquely antero-inferiorly through the posterior part of the gland and terminate in openings on the floor of the prostatic urethra. Avascular rectovesical fascia separates the posterior surface of the prostate from the anterior wall of the rectum.

The narrow, convex anterior surface of the prostate lies about 2cm behind the symphysis pubis, but is separated from it by the prostatic venous plexus and adipose tissue which occupy the retropubic space. Puboprostatic ligaments join the upper border of the anterior surface to the symphysis pubis.

The inferolateral aspects of the prostate present two prominent, rounded surfaces. The anterior portions of the levatores ani (levatores prostatae) embrace the lateral walls of the prostate, but are separated from them by the prostatic venous plexus and pelvic fascia.

The prostate gland, itself, is immediately enveloped in a thin, but firm, fibroelastic capsule which is structurally continuous with the fibromuscular element of the gland. External to this capsule, and distinct from it, is a dense fibrous sheath which surrounds the entire gland and is continuous with the supporting ligaments of the prostate. Passage of the urethra and the ejaculatory ducts through the substance of the prostate further secures its anatomical position.

Although the concept of anatomical lobation in the prostate was originally introduced by Lowsley (1912), the regional morphology of this organ still remains a controversial issue (Elbadawi, 1979a; Aumüller, 1979;

Gray, 1980). Not only are there disagreements regarding the existence of anatomical subdivisions in the prostate (Huggins and Webster, 1948), but even among those who support the concept of lobation, there are divergent views on the boundaries of the subdivisions (McNeal, 1968, 1972, 1975b, 1980a; Franks, 1975; Tisell and Salander, 1975) which have been further confused by a lack of uniformity in terminology (McNeal, 1972; 1980a). Comprehensive reviews of the current concepts in prostatic regional morphology have been presented recently by Aumüller (1979) and McNeal (1972, 1980a). However, for the purpose of the present study, it is suffice to say that the normal human prostate does not exhibit a distinctly segmented body as seen in the prostate glands of many lower mammals.

Arterial blood supply to the prostate is derived primarily from the anterior divisions of the internal iliac artery (Patil, 1979; Aumüller, 1979; Spring-Mills and Hafez, 1980). Branches of this vessel join to form the main prostatic artery which subsequently divides into urethral and capsular groups of vessels. The periurethral region of the prostate receives urethral blood vessels, while most of the parenchyma is supplied by capsular vessels. The apex of the gland receives both capsular and urethral blood vessels, but these do not anastomose freely (Patil, 1979).

Prostatic veins form the venous plexus of Santorini which is embedded in the fibrous sheath of the prostate, particularly on the anterior and lateral surfaces at the base of the gland. Drainage from the venous plexus flows into the internal iliac veins. However, valveless venous communications between the prostatic plexus and the

extradural venous plexuses are of pathological significance in the dissemination of prostatic cancer, via retrograde venous spread, to the lumbar and sacral spine, and the pelvic bones (Patil, 1979; Muir, 1980).

Lymph vessels are abundant in the human prostate and drain primarily into the external and internal iliac, and sacral lymph nodes. Lymphatic drainage is also of pathological importance in the metastatic spread of prostatic cancer (Patil, 1979; Spring-Mills and Hafez, 1980; Muir, 1980).

Autonomic innervation of the prostate is supplied by both the parasympathetic and sympathetic systems (Patil, 1979; Aumüller, 1979; Elbadawi and Goodman, 1980).

Histologically, the prostate is composed of fibromuscular and glandular elements which have been described in detail by Aumüller (1979) and Spring-Mills and Hafez (1979, 1980). The prostatic capsule consists of regularly arranged rows of collagen, fibroblasts, elastic fibres and smooth muscle cells, and gives rise to septa which extend inward, embedding the glandular tissue in a meshwork of fibromuscular stroma. Smooth muscle cells and fibroblasts are the most abundant cell types in the stroma, but the cellularity of the interstitial tissue varies considerably throughout the prostate. Posterior to the urethra, in the region of the apex, the stroma forms a loose network which increases in density toward the base of the gland. Anteriorly, the stromal tissue is very dense and this region is often devoid of glandular tissue.

The glandular element of the prostate is composed of 30 to 50 branching, tubuloalveolar glands of various shapes and sizes, which empty into the prostatic urethra via 16 to 32

excretory ducts. The glandular alveoli are lined with a single layer of folded columnar epithelium which frequently forms papillary projections that extend far into the lumen. Simple or pseudostratified columnar epithelium lines the ducts, except near the entrance to the urethra, where it changes to transitional epithelium. Within the prostatic epithelium are two cell types: (1) tall secretory or glandular cells that line the lumina and (2) small, non-secretory basal cells which are interposed between the secretory epithelium and the basement membrane.

Typical secretory cells are columnar in shape and contain a large spherical or oval, basally-located nucleus which lacks prominent nucleoli. The fine structure of these cells exhibits organelle polarization which correlates well with the secretory function of the epithelium (Brandes et al., 1964; Brandes, 1966, 1974a; Aumüller, 1979). The basal region of the cell, which includes the nucleus, contains rough endoplasmic reticulum, secretory vacuoles and clusters of free ribosomes, while the supranuclear region is primarily occupied by elements of the Golgi apparatus, as well as some endoplasmic reticulum, secretory granules and vacuoles. In the apical region of the cell, the predominant feature is an abundance of densely packed secretory vacuoles. Mitochondria are also present throughout the cytoplasm, but occur less frequently in the apical pole. The luminal plasma membrane exhibits many microvilli and often apical blebs of cytoplasm project into the lumen, suggesting that the apocrine type of secretion occurs in the human prostate, as well as the merocrine type (Brandes et al., 1964; Brandes, 1966, 1974a).

In a normal male adult, the prostate gland continuously

secretes a thin, serous fluid (pH 6.5) which contributes numerous specialized products to the composition of seminal fluid and accounts for 15 to 30% of its total volume (Spring-Mills and Hafez, 1980; Mann and Lutwack-Mann, 1981). For a detailed list of the chemical constituents present in the human prostatic secretion, the reader is referred to reviews by Mann (1954, 1964, 1974), Price and Williams-Ashman (1961), Aumüller (1979), Ahmed et al. (1979a) and Mann and Lutwack-Mann (1981). Among the most characteristic components of human prostatic fluid is, however, the enzyme, acid phosphatase. The remarkably high concentration of acid phosphatase in the human prostate was first discovered by Kutscher and Wolbergs (1935) and later confirmed by Gutman and Gutman (1938a), who also established the clinical significance of elevated serum acid phosphatase levels in the diagnosis of metastatic carcinoma of the prostate (Gutman and Gutman, 1938b; Robinson et al., 1939). Histochemical studies subsequently demonstrated the localization of intense acid phosphatase activity in the secretory epithelium of the human prostate (Gomori, 1946; Burstone, 1958; Brandes, 1966; Kirchheim et al., 1964, 1974; Brandes and Kirchheim, 1977). In normal tissue, enzyme activity is present throughout the epithelial cytoplasm, but exhibits a preponderance toward the acinar lumen. Acid phosphatase activity has also been histochemically demonstrated in the secretory blebs and luminal secretion, indicating that the enzyme is incorporated into the prostatic secretion as part of the bleb (Kirchheim et al., 1974; Brandes and Kirchheim, 1977).

In contrast to the secretory epithelial cells, the basal cells contained within the prostatic epithelium are polygonal

in shape, do not reach the lumen of the gland, show little evidence of organelle polarization and no signs of secretory activity (Brandes et al., 1964; Spring-Mills and Hafez, 1979, 1980). In addition to a few mitochondria, some endoplasmic reticulum and a poorly developed Golgi apparatus, these cells contain a moderate number of filaments, which presumably serve a contractile function, and pinocytotic vesicles which are assumed to be involved in the exchange of materials between the glandular epithelium and the stroma. However, the principal function of the basal cells is presumed to be as reserve cells for renewal of the prostatic secretory epithelium (Dermer, 1978; Spring-Mills and Hafez, 1980).

The morphology of the human prostate gland remains relatively stable until about the fifth or sixth decade, when advancing age may be associated with prostatic atrophy (Moore, 1936; McNeal, 1968, 1975b, 1980b, 1981; Elbadawi, 1979a; Coleman, 1980) and/or pathological changes due to BPH or prostatic cancer. While the latter tend to overshadow morphological changes strictly due to aging, focal and diffuse glandular atrophy are recognized as common features of the senile prostate. Age-associated prostatic atrophy shows marked individual variability but is generally characterized by dilated alveoli lined with straight, flattened epithelium, diminished secretory activity and progressive stromal fibrosis (McNeal, 1968, 1980b). The progressive accumulation of large, lamellated concretions, known as corpora amylacea, in the glandular lumina and secretion are also a characteristic feature of aging in the human prostate gland (Elbadawi, 1979a; Spring-Mills and Hafez, 1980).

Embryogenesis of the rat prostate follows a similar

pattern of development to that of the human prostate gland. Prostatic primordia in the rat arise from the urogenital sinus epithelium as independent groups of epithelial cords which branch repeatedly and secondarily acquire a lumen (Price, 1936, 1963; Narbaitz, 1974). According to Price (1936), embryologic development of the rat prostate occurs relatively late (19.5 days) in the gestation period (22 days). However, as in the human, prostatic morphogenesis in the rat appears to be dependent upon the production of androgens by the foetal testis (Wilson, 1975; Lasnitzki and Mizuno, 1977; Bard et al., 1980; Cunha and Lung, 1979a, 1980) which occurs at 15.5 days and increases sharply at 18.5 days (Price et al., 1975). In a 19.5 day foetal rat, Price (1936, 1963) identified the following groups of prostatic cords: (1) a middle group arising from the midline of the posterior wall of the urethra craniad to the openings of the ejaculatory ducts, (2) right and left lateral groups originating from the dorsolateral walls of the urethra, caudad to the openings of the ejaculatory ducts, (3) right and left dorsal groups arising from the posterior wall of the urethra, caudad to the openings of the ejaculatory ducts, and (4) right and left ventral groups arising from the ventral wall of the urethra, caudad to the level of the prostatic utricle. Glands arising from these foci give rise to the following segments of the rat prostatic complex: the anterior prostate (coagulating gland), the lateral prostate, the dorsal prostate and the ventral prostate, respectively (Price, 1963).

Unlike the human prostate, the epithelial cords of the rat prostate are undifferentiated at birth and do not

begin to undergo lumenization until the first week of post-natal life (Price, 1936). However, the process of lumenization follows a similar pattern to that in the human prostate, proceeding from the entrance of the glands into the urethra toward the periphery. Also, in contrast to the human prostate, the rat prostate does not exhibit evidence of maternal oestrogenic effects during normal foetal development (Price, 1963). Postnatally, the rat prostate is characterized by rapid growth and by 25 days it is structurally similar to the normal adult gland, although sexual maturity does not occur until about 35 days after birth (Price, 1936).

The gross anatomy of the normal adult rat prostate has been thoroughly described by Franks (1967), Levy and Fair (1973), Hebel and Stromberg (1976) and Jesik et al. (1982). Unlike the human prostate, the rat prostate consists of four pairs of anatomically distinct lobes: (1) the anterior prostate is composed of two lobes which are attached to the ventromedial margins of the seminal vesicles by a common connective tissue sheath, (2) the ventral prostate consists of two symmetrical, pear-shaped lobes which embrace the urethra on the ventromedial side of the bladder, (3) the lateral prostate lies posterior to the urethra at the neck of the bladder and is composed of two lobes which embrace, and form a continuous mass with, the paired lobe of (4) the dorsal prostate. Although the dorsal and lateral prostatic lobes are separate entities (Price and Williams-Ashman, 1961; Price, 1963; Franks, 1967; Jesik et al., 1982), they are often referred to collectively as the "dorsolateral" prostate (Hebel and Stromberg, 1976) because of their close proximity.

On the basis of the site of origin of the prostatic

primordia and the anatomical position of the tubules, Price (1963) proposed the following (Table 1.1) homologies between the rat prostatic complex and the human prostate, as described by Lowsley (1912).

Table 1.1 Homologies between the prostatic lobes in the rat and man

Rat	Man
Anterior prostate	Middle (or median) lobe
Lateral prostate	Lateral lobes
Dorsal prostate	Dorsal (or posterior) lobe
Ventral prostate	———

Since the rat ventral prostate and the anterior lobe of the human prostate differ in their site of origin from the ventral wall of the urethra, relative to the openings of the ejaculatory ducts and prostatic utricle, Price (1963) concluded that the two lobes are not homologous. However, in view of the current controversy surrounding the regional morphology of the normal human prostate, which includes a general tendency to deny the significance of Lowsley's (1912) concept of lobation in the adult gland (McNeal, 1972, 1980a), it appears that such interspecies comparisons require further clarification.

While the various segments of the rat prostatic complex are structurally similar in that they are all composed of tubuloalveolar glands, lined with a single layer of secretory epithelium and separated by fibromuscular stroma, the histological characteristics of each segment differ, as does the chemical composition of the segments

and their secretions (Moore et al., 1930; Price and Williams-Ashman, 1961; Brandes, 1966, 1974a; Mann, 1954, 1964, 1974; Aumüller, 1979). However, as in the majority of experimental studies of the rat prostate, the ventral prostate (Plate 1.1) is the subject of the present investigation and will therefore be considered in greater detail.

The normal histology of the adult rat ventral prostate was described by Moore et al. (1930) and has been discussed in extensive reviews by Franks (1967), Cavazos (1975), Aumüller (1979) and Jesik et al. (1982). The glandular alveoli and ducts are lined with straight or slightly folded, simple columnar epithelium and are separated by fine strands of fibromuscular stroma. In contrast to the human prostate, the interacinar tissue in the rat ventral prostate is very sparse and consists primarily of fibroblasts, with some smooth muscle cells (Flickinger, 1972). Undifferentiated cells are also present in the stromal tissue of the rat ventral prostate (Flickinger, 1972), whereas few comparable cells are evident in the stroma of the human prostate (Spring-Mills and Hafez, 1980). Stereological analyses have shown that the interacinar tissue comprises about 25% of the total volume of the rat ventral prostate (Bartsch, 1977; Bartsch and Rohr, 1982), whereas in the normal human prostate, stromal tissue occupies 45 to 55% of the gland (Bartsch et al., 1979; Bartsch and Rohr, 1982). As in the human prostate, however, the glandular epithelium in the rat ventral prostate is composed of tall secretory cells which line the lumina and small, non-secretory basal cells wedged between the secretory epithelium and the basement membrane. In the rat ventral prostate, a typical secretory epithelial

Plate 1.1 Transverse section through the proximal part of the urethra and the prostate gland of a sexually-mature (4-6 months old) albino rat. The ventral prostate is composed of two distinct lobes (VP) which embrace the ventrolateral aspects of the urethra (U) and are separated by the dorsolateral prostate (DLP) and fibromuscular tissue which lies ventral to the urethra. Haematoxylin & Eosin. X20.


DLP
VP
VP
U

cell is columnar in shape, contains a large round or oval, basal nucleus with one or two prominent nucleoli, and exhibits a distinct clear (ie. negatively-stained) zone in the supranuclear cytoplasm, which corresponds with the position of the Golgi apparatus (Moore et al., 1930). Ultrastructural studies have shown that the distribution of organelles in the secretory cells of the rat ventral prostate is similar to that in the human, although the rat exhibits more extensive development of the endoplasmic reticulum and Golgi apparatus (Harkin, 1957, 1961; Brandes and Groth, 1961; 1963; Brandes, 1966, 1974a; Helminen and Ericsson, 1970; Cavazos, 1975; Aumüller, 1979). Also, the merocrine mode of secretion appears to be the primary form of protein release in the rat ventral prostate (Flickinger, 1974). Histochemically, the secretory epithelium exhibits positive acid phosphatase activity (Brandes et al., 1962; Brandes, 1963, 1966; Müntzing, 1972; Paris and Brandes, 1974; Brandes and Kirchheim, 1977), but the intensity of the reaction is considerably less than in the human prostate (Brandes, 1966; Müntzing, 1972). However, as in the human prostate, the intracytoplasmic distribution of acid phosphatase in the rat ventral prostate is both lysosomal and secretory (Vanha-Perttula et al., 1972; Helminen et al., 1975; Tenniswood et al., 1976).

Advancing age in the rat, like in the human, is accompanied by morphological changes in the prostate which show marked individual variability (Mainwaring and Brandes, 1974; Burek, 1981). While some areas of the gland may exhibit atrophic changes, characterized by dilated alveoli lined with flattened epithelium, other regions of the epithelium appear hyperplastic and still others appear

normal. Glandular atrophy is also accompanied by decreased secretory activity and often the alveolar lumina contain concretions which resemble the corpora amylacea of the aged human prostate, but without the characteristic laminations (Burek, 1981). The most striking age-related change in the ultrastructure of the rat ventral prostate is a progressive accumulation of pigment granules, which show characteristics of both lysosomes and lipofuscin pigment (Harkin, 1957, 1961; Brandes, 1963, 1966; Mainwaring and Brandes, 1974). With increasing age these deposits become abundant particularly in the supranuclear and basal regions of the cell, and in rats greater than two years of age they may occupy a large proportion of the cytoplasm (Mainwaring and Brandes, 1974). Arcadi (1959) reported a similar accumulation of PAS-positive granules in the prostate glands of aging rats. Histochemical studies have further shown that, with age, the distribution of acid phosphatase activity in the secretory epithelium of rat ventral prostate also becomes concentrated in the supranuclear region (Brandes, 1963, 1966; Paris and Brandes, 1974; Mainwaring and Brandes, 1974).

1.2 INTRODUCTION TO THE ANDROGENS

While the rat and human prostate glands exhibit distinct morphological, biochemical and pathological differences, both organs share a common dependence upon androgens for their normal development and growth, and for the maintenance of their structural and functional integrity. Moreover, it is this basic similarity which has been exploited in the widespread use of normal rat prostate as a model organ in prostate research.

Androgens (Greek "andros", male) have classically been

defined as steroid hormones which are capable of stimulating the development of male secondary sex characteristics. All native androgens are 19-carbon (C_{19}) steroid hormones and have a common basic molecular structure (Fig. 1.1) consisting of a 17-carbon tetracyclic skeleton, the cyclopentanoperhydrophenanthrene nucleus, with two angular methyl groups which contribute carbon atoms 18 and 19, respectively. Individual androgen molecules differ from each other in the structure and spatial arrangement of their polar substituents.

1.2.1 Androgen Biosynthesis and Transport

Androgen biosynthesis in the male adult occurs primarily in the testis and, to a limited extent, in the adrenal cortex. The principal testicular androgen is testosterone and it accounts for more than 95% of the total testosterone in normal adult men, the remainder being derived from peripheral metabolism of other testicular or adrenal steroids (Lipsett, 1975).

The biosynthesis of testosterone has been discussed in comprehensive reviews by Eik-Nes (1975b) and Ewing and Brown (1977). Briefly, testosterone is synthesized from cholesterol in the interstitial cells of Leydig under the direct stimulation and control of LH (lutenizing-hormone), a gonadotrophic hormone secreted by the anterior pituitary in response to the hypothalamic gonadotrophin, LH-RF (lutenizing-hormone releasing factor). Testosterone biosynthesis is primarily regulated by a negative feedback system whereby the level of circulating testosterone controls the secretion of LH-RF which, in turn, regulates the release of LH from the anterior pituitary. However, other factors

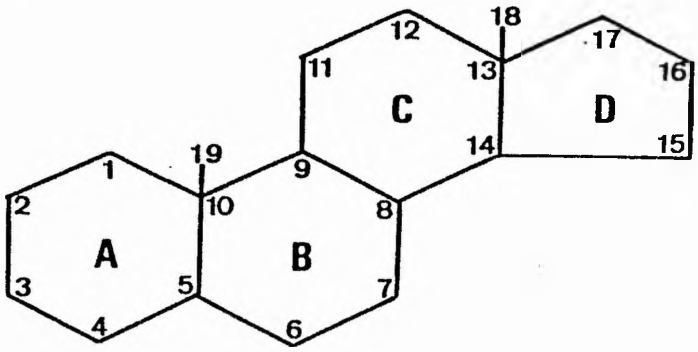


Fig. 1.1. The basic molecular structure of androgens indicating the carbon-atom numbering system used for steroid hormones. The rings of the cyclopentanoperhydrophenanthrene nucleus are designated A, B, C, D. Carbon-atoms at positions 18 and 19 are derived from angular methyl groups.

which include age, light, season, temperature and diet, and which are often species-specific, also influence the secretory activity of the hypothalamus and testis (Price and Williams-Ashman, 1961).

Testosterone gains access to the systemic circulation via the spermatic veins and is transported either in the form of complexes with plasma binding proteins or in the free (unconjugated) form (Jung et al., 1975; Eik-Nes, 1975b; Mainwaring, 1977; Menon and Walsh, 1979). In human plasma, the majority of circulating testosterone is bound to a high affinity β-globulin, known as TEBG (testosterone-estradiol binding globulin; also designated as SHBG, sex hormone binding globulin or SBP, sex steroid binding plasma protein) and with lower affinity to transcortin (CBG, corticosteroid binding α_2-globulin) and plasma albumin (Jung et al., 1975; Menon and Walsh, 1979). Free testosterone represents less than 10% of the total plasma concentration (Mawhinney and Belis, 1976). In the rat, however, TEBG is absent (Jung et al., 1975; Mainwaring, 1977) and plasma testosterone binds primarily to the corticosteroid binding α_2-globulin, CBG (Mainwaring, 1977).

At present, the process by which testosterone enters androgen-dependent target organs, such as the prostate, is incompletely understood, but it is believed to be by passive, or possibly facilitated, diffusion of the free steroid (Eik-Nes, 1975b; Moore and Wilson, 1975; Mainwaring, 1977; Aumüller, 1979; Farnsworth, 1980). Hence, it is the free moiety of testosterone which appears to assume biological importance at the cellular level. The association of testosterone with plasma binding proteins may, therefore, be more important in regulating the amount of free testosterone available to

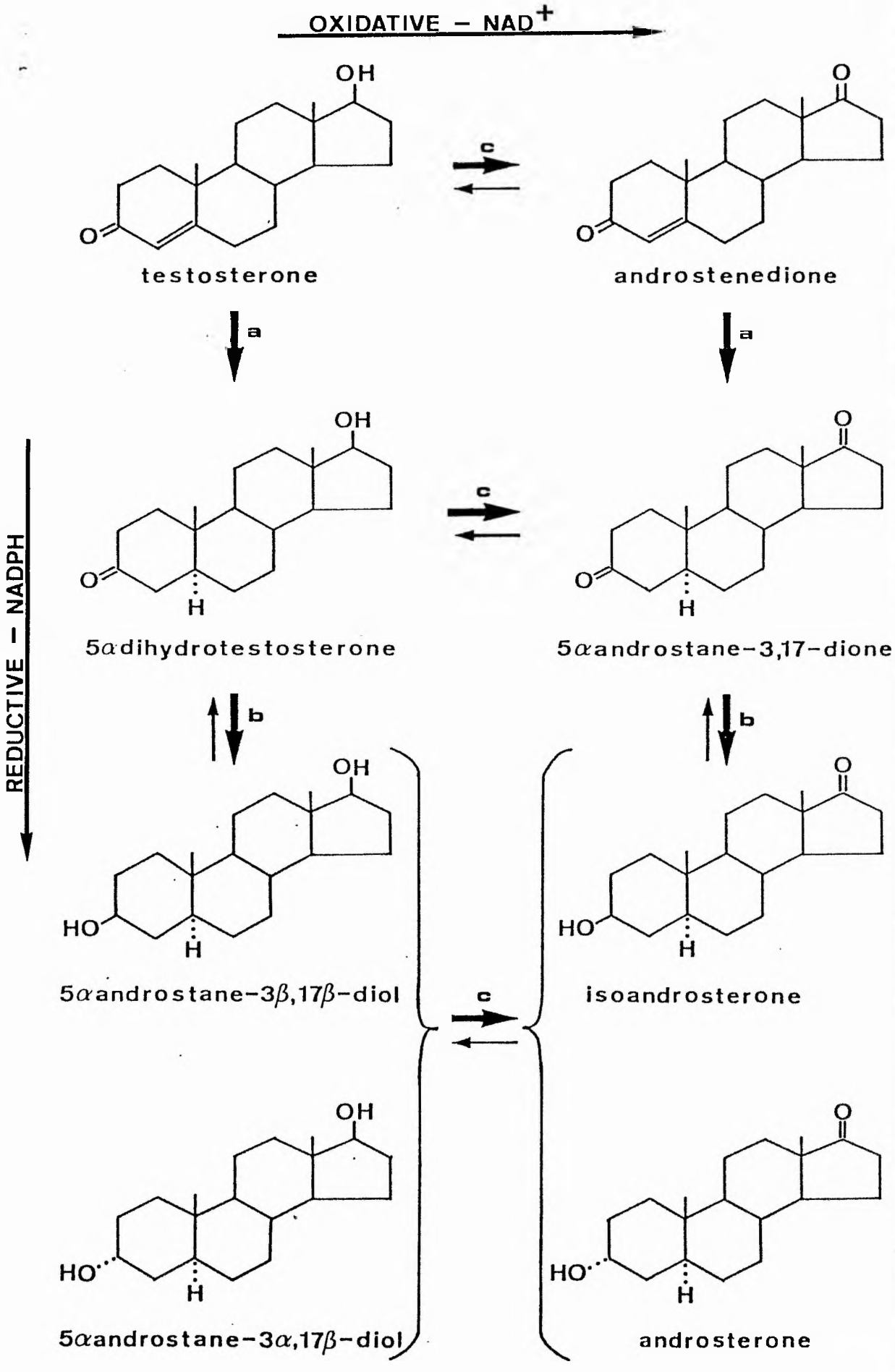
target organs, rather than as a mechanism of androgen transport (Lasnitzki and Franklin, 1972; Eik-Nes, 1975b; Lasnitzki, 1976). Furthermore, these binding proteins may serve to prevent indiscriminate androgenization and to protect the hormone from premature destruction by peripheral metabolic processes (Mainwaring, 1977).

1.2.2. Testosterone Metabolism in the Prostate

In both the rat ventral prostate and the human prostate gland, testosterone is subjected to extensive metabolism (Fig.1.2) (Farnsworth and Brown, 1963; Ofner, 1968; Bruchovsky and Wilson, 1968a, 1968b; Anderson and Liao, 1968; Siiteri and Wilson, 1970; Ofner et al., 1974; Moore and Wilson, 1975; Wilson, 1975; Eik-Nes 1975a; Mainwaring 1977; Aumüller 1979). The major intracellular metabolite, 5α-dihydrotestosterone, is formed by a rapid and irreversible reaction catalyzed by the membrane-bound enzyme, 5α-reductase (NADPH-dependent-Δ^4-3-ketosteroid-5α-oxidoreducatse). Since this metabolite is, itself, a potent androgen and the principal steroid retained by prostate cell nuclei (Bruchovsky and Wilson, 1968a, 1968b; Anderson and Liao, 1968; Tveter and Attramadal, 1969; Tveter and Aakvaag, 1969; Fang et al., 1969; Sar et al., 1970), it is generally agreed that 5α-dihydrotestosterone is the "active" form of testosterone in the prostate.

According to the current model for the mechanism of action of androgens proposed by Mainwaring (1977), the androgenic effect of testosterone is mediated by a sequence of molecular events which are related to the intracellular metabolism of testosterone and the presence of specific androgen receptor proteins (Liao et al., 1975; Liao, 1976, 1977; Mainwaring, 1977; Ahmed et al., 1979a, 1979b; Aumuller, 1979; Rennie and Bruchovsky, 1980). This model presumes that: (1) after entering the cell, testosterone is converted

Fig. 1.2 Pathways of testosterone metabolism in the prostate.
a = 5α-reductase; b = 3α-hydroxysteroid dehydrogenase;
c = 17β-hydroxysteroid dehydrogenase (Farnsworth, 1980).


OXIDATIVE – NAD+
REDUCTIVE – NADPH
testosterone
androstenedione
5αdihydrotestosterone
5αandrostane-3,17-dione
5αandrostane-3β,17β-diol
isoandrosterone
5αandrostane-3α,17β-diol
androsterone
a
b
c
OH
HO
O
H

to 5α-dihydrotestosterone, thereby minimizing the intracellular concentration of testosterone and maintaining the downhill gradient for the diffusion of testosterone into the target cell, (2) 5α-dihydrotestosterone is selectively bound with high affinity to a cytoplasmic receptor protein, (3) the resultant complex, after undergoing an activation step which modifies its stereochemical configuration, is translocated to the nucleus where it is bound to nuclear acceptor proteins and (4) binding of this complex presumably activates metabolic processes (transcription, translation, DNA synthesis and mitosis) responsible for the androgenic response. This model has gained wide acceptance through the discovery of cytoplasmic and nuclear androgen receptor proteins, which are physiochemically similar in both the rat and human prostate glands (Liao et al., 1974, 1975; Liao, 1976, 1977; Karr and Sandberg, 1979; Aumüller, 1979; Rennie and Bruchovsky, 1980; Farnsworth, 1980; de Voogt, 1982). However, the mechanism by which nuclear binding of the 5α-dihydrotestosterone complex activates the metabolic events involved in the androgenic response is, as yet, unknown.

1.2.3 Effects of Androgen Deprivation and Replacement on the Rat Prostate

While the isolation and identification of testosterone did not occur until 1935 (David et al., 1935), the fundamental relationship between the testis and the structural and functional status of the prostate was recognized as early as 1792 by John Hunter (1792) in his classic description of post-castration atrophy in the male accessory sex glands. The cytologic effects of castration were first studied by

Moore et al. (1930) in the rat prostate, and while only the ventral prostate will be considered here, castration induces similar retrogressive changes throughout the various segments of the rat prostatic complex (Moore et al., 1930; Price and Williams-Ashman, 1961; Cavazos, 1975; Aumüller, 1979). Within four days of castration the secretory epithelium in the rat ventral prostate is reduced in height and the supranuclear clear zone disappears. This is accompanied by a reduction in the size of the glandular alveoli and the more prominent appearance of the interacinar tissue. On subsequent days these changes become more pronounced and by ten days the Golgi apparatus begins to fragment. At twenty days post-castration atrophy is maximal: the secretory epithelium is a fraction of its normal height, the basement membrane has disappeared, epithelial cell nuclei are pyknotic and lack nucleoli, the Golgi apparatus is reduced to granules and the glandular alveoli are shrunken, while the proportion of stromal tissue appears to have increased (Moore et al., 1930). Korenchevsky and Dennison (1935) suggested that the increase in stromal tissue following castration may only be apparent with respect to reductions in the proportion of other tissue components. However, more recent histoquantitative analyses have shown that the volume of interacinar tissue does increase, while that of the epithelium and lumen decrease (Arvola, 1961; Romppanen et al., 1980; Huttunen et al., 1981).

Post-castration atrophy is also associated with extensive alterations in the fine structure of the secretory epithelium of the rat ventral prostate (Harkin, 1957, 1961; Brandes et al., 1962; Brandes and Groth, 1964; Brandes, 1966, 1974b; Helminen and Ericsson, 1971; Kerr and Searle, 1973; Cavazos, 1975; Brandes and Kirchheim, 1977; Aumüller, 1979).

Within 24 to 48 hours of castration, the rough endoplasmic reticulum undergoes transient dilation (Harkin, 1957) followed by gradual collapse and fragmentation of the extensive system of cisternae. Subsequently, the degenerate rough endoplasmic reticulum, as well as the Golgi apparatus, mitochondria, ribosomes and secretory vacuoles, become sequestered in autophagic vacuoles, resulting in the progressive depletion of subcellular organelles (Brandes, 1974b; Brandes and Kirchheim, 1977). Histochemical studies have also shown that a marked shift in the intracytoplasmic distribution of acid phosphatase activity accompanies involution of the glandular epithelium (Brandes, 1966, 1974b; Brandes and Kirchheim, 1977). In contrast to the diffuse distribution of acid phosphatase positive granules in the secretory epithelium of normal rat ventral prostate, enzyme activity appears in the form of large conglomerates in castrated animals. Localization of acid phosphatase activity within the autophagic vacuoles of atrophic prostatic glandular epithelium (Helminen and Ericsson, 1971) implicates the participation of lysosomal enzymes in the degradation process (Helminen and Ericsson, 1971; Brandes, 1974b; Paris and Brandes, 1974).

Depletion of the cytoplasmic organelles in castrated rat ventral prostate is accompanied by a rapid decline in RNA and protein synthesis, with resultant loss of secretory activity, and is presumably responsible for marked reductions observed in epithelial cell size (Brandes, 1966, 1974b; Helminen and Ericsson, 1971). DeKlerk and Coffey (1978) have shown that in rat ventral prostate, castration induces an 85% decrease in epithelial cell size, as well as a 92% reduction in total epithelial cell number. Similarly, Lesser and

Bruchovsky (1973) demonstrated an 85% reduction in the number of nuclei isolated from rat ventral prostate 14 days after castration, indicating that post-castration regression of the prostate is associated with significant cell loss. Morphological studies have further shown that castration-induced involution of the rat prostate is accompanied by extensive epithelial cell loss through apoptosis (Kerr et al., 1972; Kerr and Searle, 1973; Stiens and Helpap, 1981). Concomitant with reductions in both epithelial cell size and number in castrated rat ventral prostate is a cessation of cell renewal (Tuohimaa and Niemi, 1974; Tuohimaa, 1980). Within 24 hours of castration, DNA synthesis declines dramatically and by day 6 reaches a negligible level (Lesser and Bruchovsky, 1973; Bruchovsky et al., 1975; Bruchovsky and Lesser, 1976). Grossly, these changes in cell size and number are reflected by a marked decrease in tissue mass. Following castration, the rat ventral prostate rapidly atrophies and within two weeks represents only a small fraction of its original weight (Burkhart, 1942; Saunders, 1963; Coffey et al., 1968; Lesser and Bruchovsky, 1973; Kerr and Searle, 1973; Tuohimaa and Niemi, 1974; Bruchovsky et al., 1975; Tuohimaa, 1980; Huttunen et al., 1981 ; Ehrlichman et al., 1981).

Early experimental studies on androgen replacement therapy clearly demonstrated that post-castration atrophy of the rat prostate is prevented or rapidly repaired by the administration of bull testis extracts or exogenous testosterone (Moore et al., 1930; Moore and Price, 1938). Moore and colleagues found that prostate glands from 13 to 100 day castrated rats were histologically indistinguishable from the normal intact gland within 20 days of androgen

treatment. Later, Burkhart (1942) studied the earliest histologically detectable effects of testosterone on castrated rat prostate and showed that increases in both epithelial cell size and mitotic activity were evident within two days of androgen treatment. Epithelial cell hypertrophy and enlargement of the cell nuclei were detected 23 hours after testosterone stimulation, while increases in mitotic activity were observed after 35 hours and reached maximum levels at about 43 hours. Subsequent studies have shown that the first biochemical manifestation of androgen action in castrated rat ventral prostate is a rapid increase in RNA and protein synthesis, which follows within a few hours of hormonal stimulation (Coffey et al., 1968; Coffey, 1974; Mainwaring, 1977; Aumüller, 1979). Evidence of restitution of the ultrastructural and functional integrity of castrated rat prostate also become apparent within 24 hours of testosterone treatment (Brandes, 1974b). In contrast, the effect of androgens on prostatic DNA synthesis in the castrated rat is a late event characterized by a latent period of 24 to 48 hours, which is followed by a surge of DNA synthetic activity that peaks on day 3 to 4 and, thereafter, declines despite the continued administration of testosterone (Coffey et al., 1968; Lesser and Bruchovsky, 1973; Coffey, 1974; Coffey and Sloan, 1975; Mainwaring, 1977; Tuohimaa, 1980). The latent period, known as the "pre-replicative phase" (Alison and Wright, 1981), represents the time interval during which androgen-induced RNA and protein synthesis occur, and which appear to be necessary for the initiation of DNA synthesis and subsequent mitosis (Mainwaring, 1977). However, the induction of prostatic DNA synthesis and cell division are refractory to

androgen stimulation unless sensitized by declining cell number and, hence, are dependent upon the time elapsed between castration and the onset of hormonal stimulation (Bruchovsky et al., 1975; Bruchovsky and Lesser, 1976; Alison et al., 1976; Alison and Wright, 1979a, 1981; Tuohimaa, 1980). Furthermore, the velocity and magnitude of the proliferative response are directly related to the time elapsed since castration (Alison et al., 1976; Alison and Wright, 1979a, 1981; Tuohimaa, 1980), as well as dependent upon the dose and potency of the androgen administered and the duration of the treatment (Saunders, 1963; Robson et al., 1965; Lesser and Bruchovsky, 1973; Tuohimaa and Niemi, 1974; Österberg and Tuohimaa, 1975; Bruchovsky et al., 1975; Bruchovsky and Lesser, 1976; Tuohimaa, 1980).

The effect of androgens on prostatic DNA synthesis in the castrated rat is typical of many mammalian systems of induced-growth in that all exhibit common, characteristic phases which have been depicted in Baserga's (1969, 1971) classical model of the cell cycle (Fig. 1.3) (Carter et al., 1972; Coffey, 1974; Coffey and Sloan, 1975). Following stimulation, the first stage of the cell cycle, termed GAP 1 or G_1, occurs and is characterized by rapid RNA and protein synthesis. Subsequently, the period of DNA synthesis, designated S phase, ensues and represents the time interval during which the cell duplicates its total DNA content. Cessation of DNA synthesis is followed by a third period, known as GAP 2 or G_2, and is marked by a series of biochemical events, notably RNA and protein synthesis, which appear to be necessary for the cell to traverse G_2 and enter mitosis, or M phase, which ultimately produces

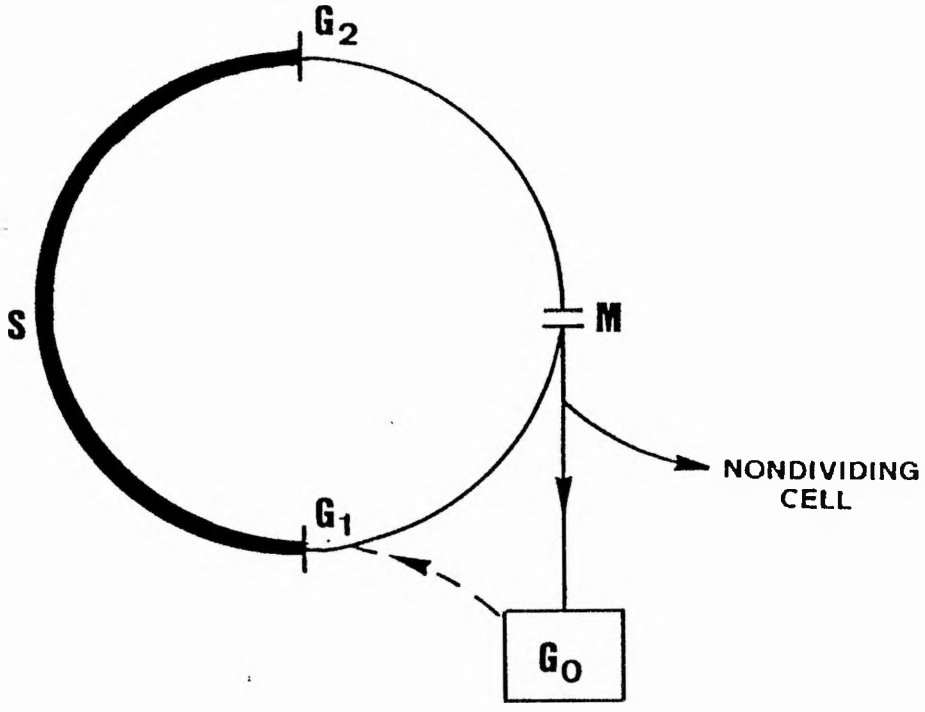


Fig. 1.3 The cell cycle. G_0 = resting phase; G_1 = pre-replicative phase; S = DNA synthesis; G_2 = post-replicative phase; M = mitosis.

two daughter cells (Baserga, 1969, 1971, 1976). After mitosis a daughter cell may remain in the cell cycle or leave the cycle to enter a population of non-proliferating cells. Such "decycling" cells may either (a) differentiate irreversibly, function and eventually die, (b) undergo immediate necrosis, or (c) enter a state of proliferative rest, termed G_0, and from which they may return to the cell cycle (Aherne et al., 1977).

In the normal, adult animal the prostate is a proliferatively quiescent tissue since most cells are in G_0 (Alison et al., 1976; Alison and Wright, 1979a, 1979b, 1981). However, after a period of androgen deprivation caused by castration, the administration of exogenous androgens stimulates a transitory increase in prostatic DNA synthesis and cell division. According to Tuohimaa (1980), the administration of androgens to castrated rodents has a dual effect on the cell proliferation kinetics of the prostate. First, androgens reduce the size of the non-proliferating (G_0) cell population, resulting in an increased growth fraction as new cells are recruited into the cell cycle (Alison and Wright, 1979a, 1981). Second, androgens enhance the velocity of cell cycling, leading to a shortening of all phases of the cell cycle (Tuohimaa and Niemi, 1968, 1974; Tuohimaa, 1980). However, despite continued androgen administration, hormone-induced prostatic cell proliferation is a transitory event which ceases once pre-castration cellularity has been restored (Bruchovsky et al., 1975; Bruchovsky and Lesser, 1976; Alison et al., 1976; McHanwell et al., 1976; Alison and Wright, 1979a, 1981; Tuohimaa, 1980). Tissue-specific growth inhibitors, such as "chalones" (Bullough, 1965; Bullough et al., 1967), have been considered as a possible growth-limiting mechanism in the prostate

(Bruchovsky et al., 1975; Bruchovsky and Lesser, 1976, Tuohimaa, 1980). Theoretically, the amount of the inhibitor produced by the normal number of cells would be sufficient to prevent cell proliferation, whereas a reduction in cell number would allow the prostate to regenerate until normal cellularity was restored (Bruchovsky et al., 1975; Bruchovsky and Lesser, 1976). Alternatively, physicochemical interactions between the fibromuscular stroma and the epithelium may be responsible for the regulation of prostatic growth (Franks et al., 1970; Müntzing and Murphy, 1977; Müntzing et al., 1979; Müntzing, 1980).. Müntzing (1980) proposes a mechanism whereby the rate of collagen synthesis controls the amount of stromal tissue which, in turn, determines the rate of epithelial cell proliferation. The hypothesis is further supported by recent evidence indicating the importance of epithelial and stromal interactions during embryogenesis of the prostate (Lasnitzki and Mizuno, 1979, 1980; Cunha and Lung, 1979b, 1980; Bard et al., 1980).

The rapid and dramatic response of the rat prostate to androgen deprivation and replacement has resulted in its use both as a classical androgen bioassay and an experimental model for studying androgen-induced growth of the prostate. While cell renewal in the prostate is clearly dependent upon androgenic stimulation, the hormonal mechanisms involved in the regulation of prostatic growth are poorly understood. Insight into the factors controlling prostatic DNA synthesis is, however, essential since this represents the uncontrolled element which produces abnormal, hyperplastic growth in the human prostate. The rate of DNA synthesis in castrated rat ventral prostate thus provides a useful parameter for assessing the effects of hormones on prostatic growth (Coffey et al., 1968; Coffey, 1974; Coffey and Sloan, 1975). In addition, this model has been extended to provide a method of assaying the anti-

proliferative potency of antiandrogenic compounds using androgen-induced DNA synthesis as an index of prostatic growth (Carter *et al.*, 1972; Sufrin and Coffey, 1973, 1975; Sloan *et al.*, 1975).

1.3 INTRODUCTION TO ORGAN AND CELL CULTURE

Organ culture, defined as "the maintenance or growth of tissues, organ primordia or the whole or parts of an organ *in vitro* in a way that may allow differentiation and preservation of the architecture and/or function" (Federoff, 1967), provides a strategy for investigating the direct effects of hormones and chemotherapeutic agents on prostatic growth. More than 50 years ago, Fell and Robison (1929) developed the classical organ culture technique, whereby fragments, or explants, of organs were grown on the surface of a coagulum of plasma and embryo extract. However use of the plasma clot method was largely replaced during the 1950's through the development of synthetic fluid media (Trowell, 1955, 1959; Waymouth, 1959, 1965; Paul, 1975) and since then, organ culture methodology has undergone numerous modifications due to the increased application of this technique to a variety of specialized tissues and biomedical problems (Foley and Epstein, 1964; Fell, 1964, 1976; Easty, 1970; Baulieu *et al.*, 1975; Hodges, 1976; Balls and Rao, 1980; Lieber and Veneziale, 1980). Current organ culture procedures primarily employ the basic technique introduced by Trowell(1954, 1959), whereby a platform of perforated metal, known as a "grid", is used to support the tissue explants at the interface between the fluid medium and gaseous atmosphere of the culture vessel.

Organ culture offers several advantages for the study of hormone-dependent tissues, such as the prostate (Lasnitzki, 1974). In contrast to *in vivo* experiments, the endocrine

environment of the tissue can be precisely defined and easily manipulated. Hence, in the isolated organ the direct effect of a single hormone, or the interaction of several compounds, can be studied under controlled and easily reproducible experimental conditions, which are devoid of systemic effects. Organ culture also preserves the architecture of the tissue, thereby enabling histological analyses of the various tissue components and their relationships, as well as permitting correlations between morphological and biochemical parameters. Preservation of the histological architecture may be of particular significance with respect to the prostate as interactions between the epithelium and stroma appear to play an important role in the normal development, growth and function of the gland (Franks et al., 1970; Franks, 1977; Müntzing and Murphy, 1977; Müntzing et al., 1979; Lasnitzki and Mizuno, 1979, 1980; Cunha and Lung, 1979b, 1980; Müntzing, 1980; Bard et al., 1980).

Clearly, organ culture is well-suited to studies of human tissue and, undoubtedly, the ideal model for prostatic cancer would utilize tissue of human origin, however, several factors limit the realization of this goal (Franks, 1977; Merchant, 1979, 1980; McNeal, 1980b). First, human prostatic tissue, particularly normal tissue, is not readily available in any quantity. While surgical intervention is relatively common in the treatment of BPH, use of the transurethral resection (TUR) procedure provides only a limited amount of tissue which is suitable for culture. Radical prostatectomy is a considerably less common therapeutic measure for treatment of BPH, or prostatic cancer, hence such tissue is rarely available. Secondly, the human prostate gland, even in its normal state, is a heterogeneous organ and this

variability is further accentuated by morphological changes due to aging and/or the development of BPH or prostatic cancer. Hence, the acquistion of replicate tissue samples is very difficult, particularly if the specimens are obtained by TUR. Thirdly, the time elapsed between procurement of the tissue and establishment of the culture may be of critical importance since the prostatic secretion contains a high concentration of enzymes, notably proteases, which may affect tissue viability. Finally, prostatic cancer is usually only detected in its advanced stages when the tumour has quite likely undergone considerable variation and selection and, therefore, may not be representative of the original tumour. Moreover, Merchant (1979) has stressed that the prior therapeutic history of the patient may also influence the tumour response in vitro.

Use of normal animals, of similar age and physiological status, as a source of prostatic tissue eliminates some of the problems and variables associated with human prostatic tissue and can provide valuable insight into the actions of hormones and chemotherapeutic agents on the prostate. During the past 30 years, the extensive work of Ilse Lasnitzki on normal rodent prostate in organ culture has clearly demonstrated the value of this experimental system for investigations into the effects of hormones, vitamins, chemotherapeutic agents and carcinogens on prostatic tissue (Lasnitzki, 1951, 1954, 1955, 1962, 1963, 1964, 1965b, 1970a, 1970b, 1974, 1975, 1976; Lasnitzki and Robel, 1969, Lieber and Veneziale, 1980). Organ culture studies of normal rat ventral prostate have shown that the tissue remains androgen-dependent and responsive in vitro. In the absence of androgens, the tissue undergoes regressive changes similar to post-castration atrophy in vivo: the

alveoli shrink, epithelial height is reduced, the supranuclear clear zone disappears, there is a loss of epithelial folding, secretory activity is diminished or absent and the proportion of stromal tissue increases (Lasnitzki, 1974). Ultrastructural alterations in the secretory epithelial cells are also similar to those observed *in vivo* following castration. Within two days of culture in androgen-free medium the rough endoplasmic reticulum collapses, the Golgi apparatus becomes disorganized and the luminal microvilli disappear. By four days, the rough endoplasmic reticulum is markedly reduced, secretory vacuoles are absent and autophagic vacuoles appear throughout the cytoplasm (Gittinger and Lasnitzki, 1972; Lasnitzki, 1974, 1975, 1976; Ichihara, 1976, 1977). Biochemical studies have also shown that the acid phosphatase activity in rat ventral prostate cultured in testosterone-free medium is greater than that in freshly excised tissue or in explants treated with testosterone (Lasnitzki *et al.*, 1966). This increased acid phosphatase activity in androgen-free cultures, along with the development of autophagic vacuoles, is consistent with the hypothesis that castration-induced involution of the prostate *in vivo* is associated with lysosomal enzymes (Lasnitzki, 1975).

The addition of testosterone to organ culture media has a well-documented effect on the maintenance of cytodifferentiation and stimulation of epithelial cell proliferation in normal rat prostate (Lasnitzki, 1965b, 1970a, 1970b, 1974, 1975, 1976; Baulieu *et al.*, 1968a, 1968b, 1969; Robel *et al.*, 1971; Gittinger and Lasnitzki, 1972; Johansson and Niemi, 1975; Johansson, 1976; Ichihara, 1976, 1977; Lieber and Veneziale, 1980; Sandberg and Kadohama, 1980). Rat ventral prostate in organ culture concentrates testosterone from the medium (Lasnitzki, 1971, 1974, 1976; Lasnitzki *et al.*, 1974) and studies of testosterone

metabolism *in vitro* have shown that it is similar to the *in vivo* conversion of testosterone to various 5α-androstane derivatives, notably 5α-dihydrotestosterone (Baulieu *et al.*, 1968a, 1968b, 1969; Robel *et al.*, 1971, 1975; Lasnitzki, 1971, 1974, 1976; Johansson, 1976). Furthermore, organ culture studies on the effects of testosterone and its metabolites on rat prostate strongly suggest that the androgenic action of testosterone is mediated via its intracellular metabolites (Baulieu *et al.*, 1968a, 1968b, 1969; Robel *et al.*, 1971; Gittinger and Lasnitzki, 1972; Lasnitzki, 1970a, 1970b, 1971, 1974, 1975, 1976). While all the metabolites exhibit some degree of androgenic activity, 5α-dihydrotestosterone is the most potent in stimulating epithelial cell proliferation, whereas 5α-androstane-3β,17β-diol effectively maintains cytoplasmic differentiation and secretory activity, but does not induce epithelial hyperplasia (Baulieu *et al.*, 1968a, 1968b, 1969; Robel *et al.*, 1971; Lasnitzki, 1970a, 1970b, 1971, 1974, 1975, 1976). Thus, Baulieu *et al.* (1968a, 1968b, 1969) suggested that 5α-dihydrotestosterone may be responsible for the regulation of cell proliferation in the rat prostate, while cytodifferentiation and secretory function are maintained by 5α-androstane-3β,17β-diol. As the human prostate exhibits a similar pattern of testosterone metabolism to that of the rat, experimental studies of rat ventral prostate in organ culture may, therefore, be considered relevant to the human gland and can provide a valuable reference for the interpretation of hormonal responses in the human prostate (Lasnitzki, 1977; Tuohimaa, 1980).

While the effects of testosterone and its metabolites on differentiation and proliferation in cultured rat prostate have been well-defined qualitatively, few studies have

attempted to quantitate the proliferative response to androgen stimulation and, among those which have, the results are often conflicting. These inconsistencies have generally been attributed to variations in the composition of the culture medium. With few exceptions, organ culture studies of rat prostate routinely employ media containing serum and, less frequently insulin, yet both of these substances are known to influence the proliferative activity of rat prostate *in vitro* and its response to androgen stimulation (Lasnitzki and Franklin, 1972, 1975; Johansson and Santti, 1973; Johansson, 1975; Donaldson and Thomas, 1976). Consequently, the full potential of this system has not been realized primarily because the design of an organ culture environment is multifaceted and, as yet, there is no uniformity in the composition of organ culture media or the experimental procedure employed. Therefore, in an attempt to establish an *in vitro* model for studying the direct effects of hormones and chemotherapeutic agents on prostatic growth, the present study investigated the proliferative activity of normal rat ventral prostate in chemically-defined organ culture using the incorporation of 5-[^{125}I]-iodo-2'-deoxyuridine (^{125}I-UdR) to monitor DNA synthesis.

The γ-emitting radioisotope, ^{125}I-UdR, is a specific precursor of DNA (Hughes *et al.*, 1964) which has been widely used in kinetic studies of prostatic tissue in organ culture (Littlewood *et al.*, 1974; Shipman *et al.*, 1975; Donaldson and Thomas, 1976; Riches *et al.*, 1976a, 1976b, 1982; Mistry *et al.*, 1982) and has proven a useful alternative to [methyl-^{3}H]-thymidine (^{3}H-TdR) for monitoring cell proliferation (Riches *et al.*, 1976a, 1976b). Although ^{125}I-UdR can be both radiotoxic and cytotoxic, it is unlikely that such effects are significant at low doses in short-term studies (Riches *et al.*, 1976a).

Moreover, reutilization problems inherent with the use of ^{3}H-TdR (Maurer, 1981) are reduced with ^{125}I-UdR (Dethlefsen, 1971, 1974) and it has the advantage over β-emitting radionucleotides in that DNA synthesis can be monitored in whole tissues without histoquantitation, extensive biochemical processing or destruction of the tissue sample (Riches et al., 1976a). Simply washing the tissue with 70% alcohol removes 98 to 99% of the unbound radioactivity (Fidler, 1970; Micklem, 1972). Furthermore, fixation in alcoholic Bouin's fluid does not alter the ^{125}I-UdR activity in cultured prostatic tissue (Riches et al., 1976a, 1976b), thus ^{125}I-UdR uptake can be monitored in fixed tissue which may be subsequently processed for histological, histochemical or histoquantitative analyses.

In the present study, the initial objective was to characterize the proliferative activity of young adult rat (4 to 6 months old) ventral prostate in chemically-defined organ culture, both in the presence and absence of testosterone, using the ^{125}I-UdR labelling technique. This parameter was then correlated with the histological response of the tissue, which was also compared with the distribution of ^{3}H-TdR labelling in autoradiographs. The specificity of ^{125}I-UdR uptake was further verified by comparing ^{125}I-UdR activity in whole explants with that of DNA biochemically extracted from replicate explants.

Variations in organ culture media (ie. $\pm$ serum and/or insulin) and methodology were also investigated for their effect on the proliferative activity of rat ventral prostate and its response to testosterone stimulation. Since the age of the tissue is also known to influence the response of rodent prostate to testosterone (Franks, 1959a; Lasnitzki, 1954, 1974), the proliferative activity of aged ($>$ 12 months old) rat ventral prostate was compared with that of younger tissue.

The established method of quantitative organ culture was then used to compare the proliferative response of young adult rat ventral prostate to testosterone with that of its major metabolites, 5α-dihydrotestosterone, androstenedione, androstanedione and 5α-androstane-3β,17β-diol. The proliferative response to 5α-dihydrotestosterone was also compared with that of its epimer, 5β-dihydrotestosterone. The ^{125}I-UdR data were then correlated with the histological response of the tissue.

Quantitative organ culture was also used to investigate testosterone-induced DNA synthesis in rat ventral prostate as a potential model for evaluating the antiproliferative actions of oestrogens, antiandrogens and hormone-cytotoxic agents. Using optimal and minimal testosterone concentrations for maximal ^{125}I-UdR uptake, the inhibitory effects of cyproterone acetate and diethylstilboestrol were investigated on the testosterone stimulated response. Similarly, the effects of Estracyt (estramustine phosphate disodium and estramustine phosphate), and its metabolite, estramustine, were compared with that of their carrier-hormone, oestradiol-17β. The effects of estramustine phosphate, estramustine and oestradiol-17β on the proliferative response stimulated by testosterone were also compared in the presence and absence of foetal calf serum, as per Høisaeter (1975b). The ^{125}I-UdR results were also compared with the histological response of the tissue.

While organ culture provides the initial step in determining the effects of hormones and chemotherapeutic agents on prostatic tissue <u>in vitro</u>, it has become increasingly apparent that to further define the actions of these compounds it is necessary to obtain pure populations of prostatic cell types for experimental manipulation. Thus, the specific effects of such compounds could be correlated with the responses of individual

cell types. Cell culture, defined as "the *in vitro* study of disaggregated cells" (Federoff, 1967), provides an ideal method for studying cellular and metabolic processes of the two major cell fractions of the prostate, namely the epithelium and stroma, in a controlled environment. Separation of these different cell types should also permit investigation of their intercellular relationships by recombination of cell populations (Chevalier *et al.*, 1980). Furthermore, the development of cell cultures of normal prostatic epithelium could serve as a model system for studies on carcinogenesis, as well as for the screening and assessment of chemotherapeutic agents which may be of potential value in the clinical management of prostatic cancer (Franks, 1977; McLimans *et al.*, 1977; Merchant, 1979; Webber, 1978, 1979, 1980). However, in order to develop such a model system it is first necessary to isolate pure populations of viable prostatic epithelial cells and confirm their epithelial origin, as well as to determine their nutrient requirements and growth potential *in vitro*. Hence, prostate cell culture research has been primarily concerned with the development of methods for the isolation, characterization and culture of prostatic epithelial cells.

Most primary cell cultures of prostatic epithelium have been established from outgrowths of tissue explants (Stonington and Hemmingsen, 1971; Schroeder *et al.*, 1971; Webber *et al.*, 1974; Lewis and Kaack, 1980; Malinin *et al.*, 1980; Clarke and Merchant, 1980; Lewis *et al.*, 1981) or from aggregates of epithelial cells derived from enzymatically (Stone *et al.*, 1976; Burleigh *et al.*, 1980; Douglas *et al.*, 1980; Webber, 1979, 1980) or mechanically (Franks *et al.*, 1970; Lechner *et al.*, 1978) dissociated tissue. However, the efficacy of such procedures in removing stromal cells, in particular fibroblasts, from "purified"

epithelial cell fractions is questionable. Hence, several studies have investigated the use of density gradients for the isolation of epithelial cells from suspensions of enzymatically-dissociated tissue (Dow and Pretlow, 1975; Helms et al., 1976; Pretlow et al., 1977; Chevalier et al., 1980; Rubenstein and Anderson, 1980; Clark et al., 1982).

In an attempt to establish a rapid, yet simple and reliable, method of obtaining pure populations of viable epithelial cells from normal rat ventral prostate, the present study investigated the use of discontinuous Percoll^{R} density gradients. Cytological examination and the histochemical demonstration of acid phosphatase activity were used to identify cells of epithelial origin. Since the demonstration of formalin-resistant acid phosphatase activity in human prostatic epithelium has been used as a histochemical marker to characterize isolated or cultured prostatic epithelial cells (Stonington et al., 1975), the formalin sensitivity of this enzyme in cryostat sections of rat ventral prostate was compared with that of human BPH in order to evaluate this method as a means of identifying epithelial cells derived from rat prostate. A preliminary evaluation of the growth potential of isolated rat ventral prostatic epithelial cells in vitro was also investigated using ^{125}I-UdR uptake and the incorporation of ^{3}H-TdR coupled with autoradiography.

CHAPTER 2

MATERIALS AND METHODS

2.1 ORGAN CULTURE

2.1.1 Animals

Male adult albino rats derived from the Wistar strain were used as tissue donors. The animals were categorized according to age into one of the following groups:

1. Young sexually mature rats from 4-6 months of age and ranging in weight from 300-500g.
2. Retired breeding rats more than 12 months of age and weighing 650-800g.

The animals were housed in a temperature (20-22°C) and light (12h light/12h dark) controlled environment, and were maintained on BP No.1 small laboratory animal diet and tap water *ad libitum*.

2.1.2 Organ Culture Technique

The basic organ culture technique used was similar to the method described by Trowell (1954, 1959) and included modifications by Riches *et al.* (1973). The animals were sacrificed by ether anaesthesia and the ventral lobes of the prostate were aseptically removed. The tissue was rinsed and decapsulated in RPMI 1640 tissue culture medium (Flow Laboratories Ltd.) supplemented with 10^{-3}M HEPES (N-2-hydroxyethyl-piperazine-N'-2-ethane sulphonic acid), 50μg ml^{-1} kanamycin (Flow Laboratories Ltd.) and 40μg ml^{-1} gentamicin (Roussel Laboratories Ltd.). To minimize trauma and avoid regenerative hyperplasia, the tissue was gently teased (Lasnitzki, 1965a) into $2mm^3$ fragments with syringe needles (No.25G). Four explants were placed onto a moistened square of cellulose acetate membrane filter (0.45μm Millipore filter;

Oxoid Ltd.) mounted on a stainless steel grid in a 30mm plastic Petri dish (Sterilin Ltd.). In designated cultures the explants were placed either directly onto the grid or onto the bottom of the culture dish. Waymouth's MB 752/1 tissue culture medium (Flow Laboratories Ltd.) containing 50μg ml^{-1} kanamycin, 40μg ml^{-1} gentamicin and 2mM L-glutamine (Flow Laboratories Ltd.) was dispensed in 3ml aliquots such that the medium formed a meniscus with the undersurface of the grid. Appointed cultures received antibiotic and L-glutamine supplemented medium containing 3μg ml^{-1} bovine insulin (Sigma Chemical Company Ltd.) and/or foetal calf serum (Flow Laboratories Ltd.) at concentrations of 5, 10 or 20%. The culture dishes were randomly arranged in 90mm plastic Petri dishes (3 or 4, 30mm dishes per 90mm dish) and stacked in McIntosh and Fildes jars lined with paper towel dampened with double distilled water. The jars were flushed with 95% oxygen and 5% carbon dioxide for 30 minutes at a flow rate of 150cc minute^{-1}, then sealed and incubated at 37°C for a period of 4-6 days. Unless otherwise stated, the medium was renewed every second day and the jars regassed.

2.1.3 Androgens

2.4 x 10^{-2}M solutions of the following androgens (Sigma Chemical Company Ltd.) were prepared and serially diluted in absolute ethanol (Aristar grade; British Drug House Ltd.). Directly before use the alcohol solutions were diluted 1:10 with Waymouth's medium such that the addition of 50μl to 3ml of culture medium produced the desired final concentrations. Control cultures received an equal volume of absolute ethanol diluted 1:10 with

Waymouth's medium.

1)	Testosterone	Fig. 2.1	Sigma T-1500
2)	5α-dihydrotestosterone	Fig. 2.2	Sigma A-8380
3)	Androstenedione	Fig. 2.3	Sigma A-9630
4)	Androstanedione	Fig. 2.4	Sigma A-8255
5)	Androstane-3β,17β-diol	Fig. 2.5	Sigma A-7630
6)	5β-dihydrotestosterone	Fig. 2.6	Sigma E-5376

2.1.4 Hormone-Cytotoxic Compounds

The following estramustine compounds were kindly supplied by AKTIEBOLAGET LEO Research Laboratories, Helsingborg, Sweden. With the exception of estramustine phosphate sodium (LS 299Z), the compounds were prepared as 1 x 10^{-2}M solutions in absolute ethanol and serially diluted such that the addition of 10μl to 3ml of culture medium produced the desired final concentrations. Following the same procedure, and in accordance with its physiochemical properties, estramustine phosphate sodium was prepared in 0.15M saline. Control cultures received equal volumes of either absolute ethanol or saline.

1)	Estramustine phosphate sodium	Fig. 2.7	LS 299Z
2)	Estramustine phosphate	Fig. 2.8	LS 299
3)	Estramustine	Fig. 2.9	LS 275

2.1.5 Oestrogens

1 x 10^{-2}M solutions of the following oestrogens (Sigma Chemical Company Ltd.) were prepared and serially diluted in absolute ethanol such that the addition of 10μl to 3ml of culture medium produced the required final concentrations.

1)	Oestradiol-17β	Fig. 2.10	Sigma E-8875

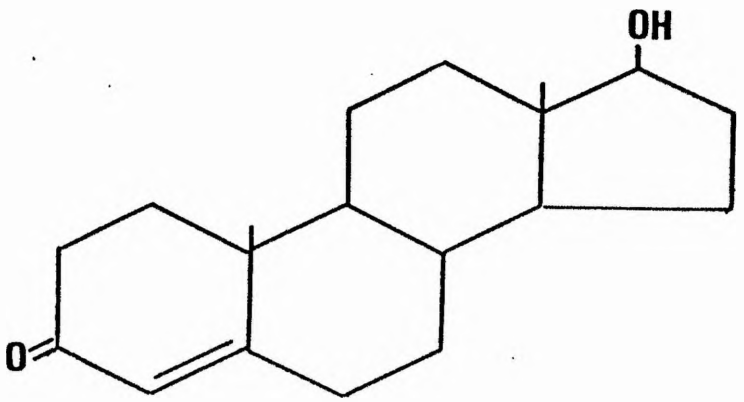


Fig. 2.1. Testosterone (MW 288.4)

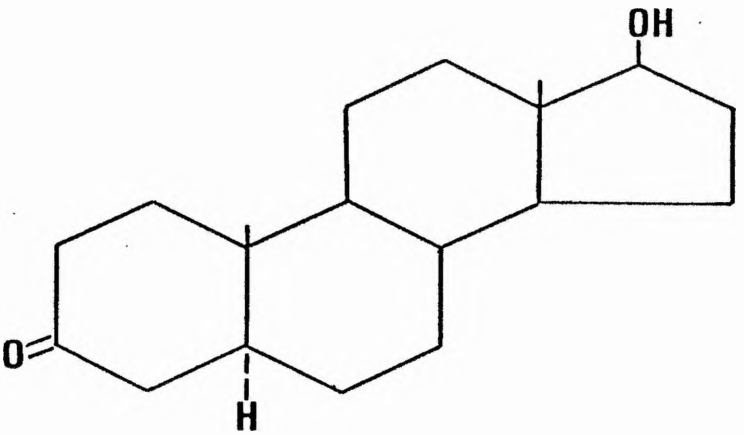


Fig. 2.2. 5α-dihydrotestosterone (MW 290.4)

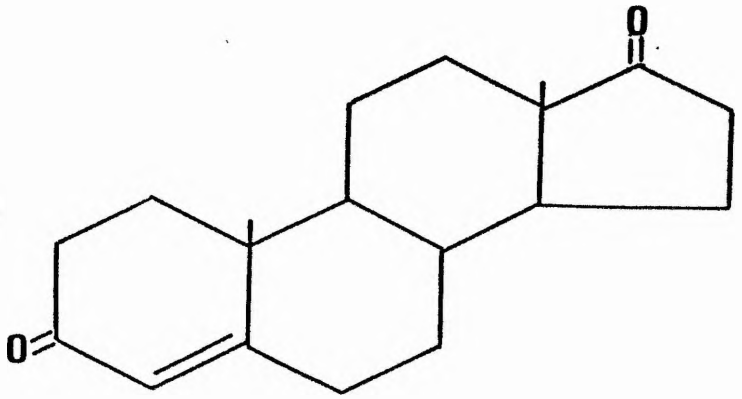


Fig. 2.3. Androstenedione (MW 286.4)

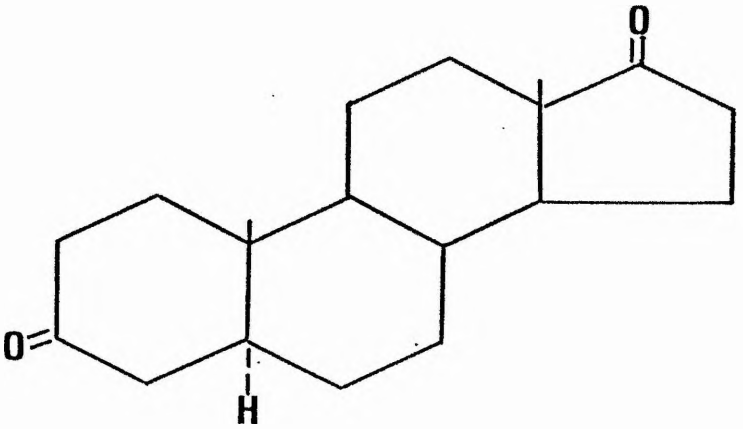


Fig. 2.4. Androstanedione (MW 288.4)

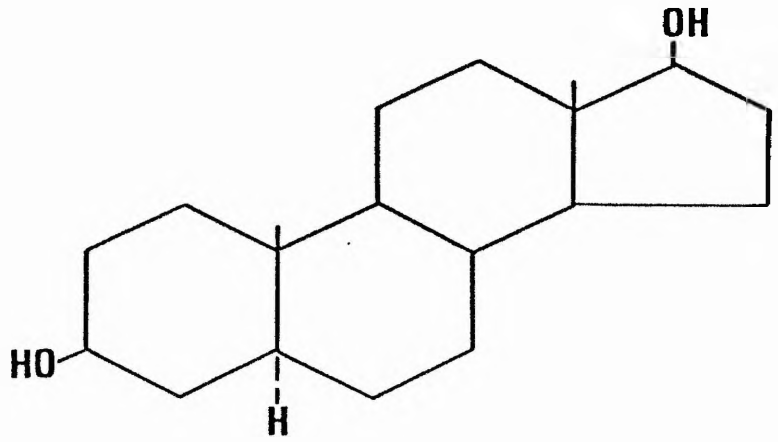


Fig. 2.5. Androstane-3β,17β-diol (MW 292.5)

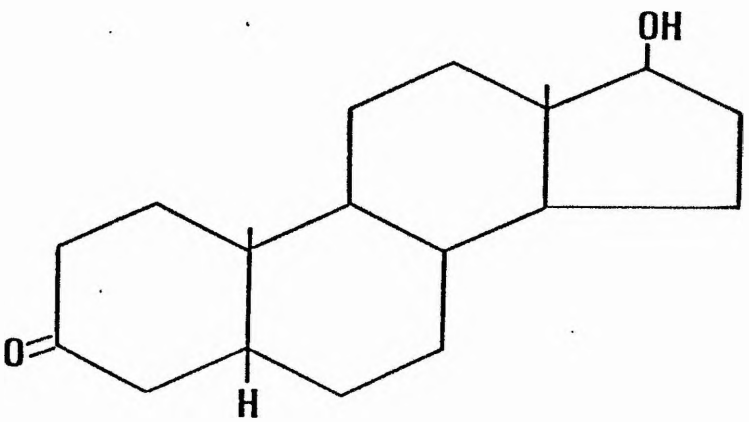


Fig. 2.6. 5β-dihydrotestosterone (MW 290.5)

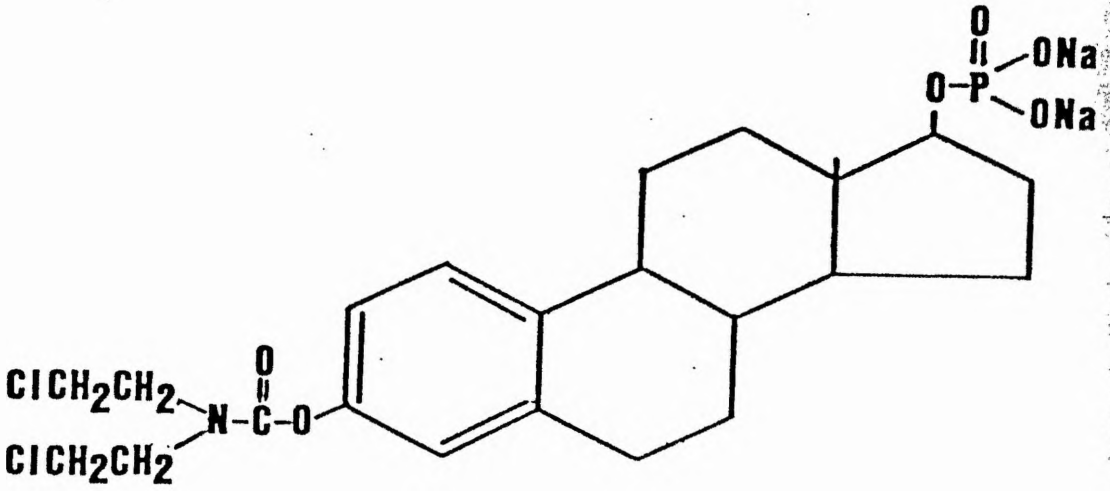


Fig. 2.7. Estramustine phosphate sodium (MW 564.39)

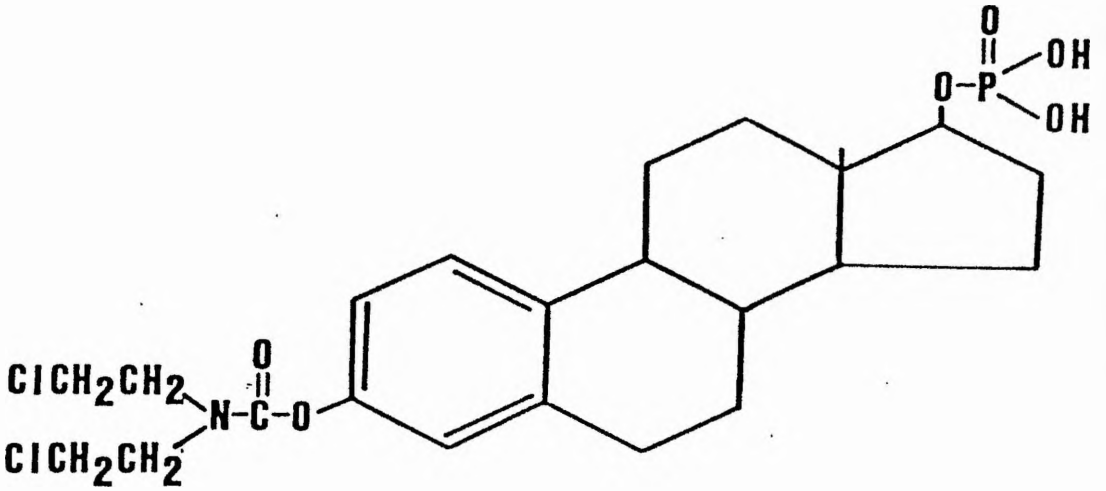


Fig. 2.8. Estramustine phosphate (MW 520.39)

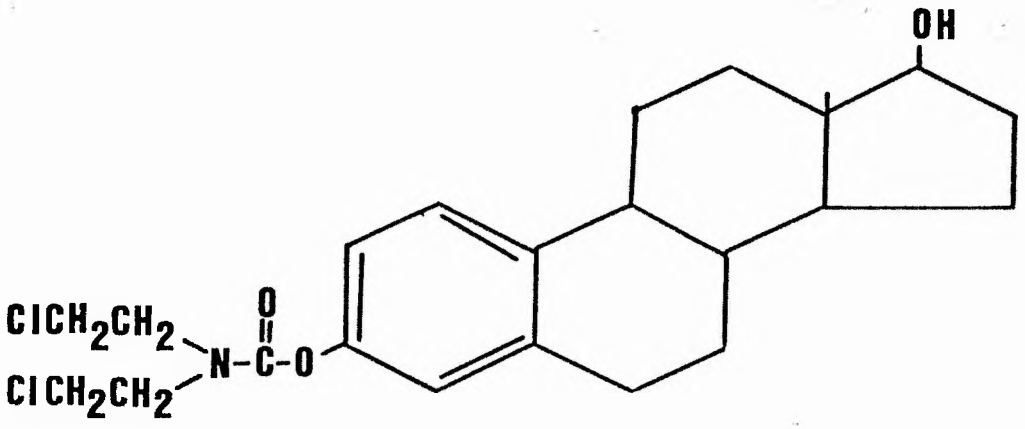


Fig. 2.9. Estramustine (MW 440)

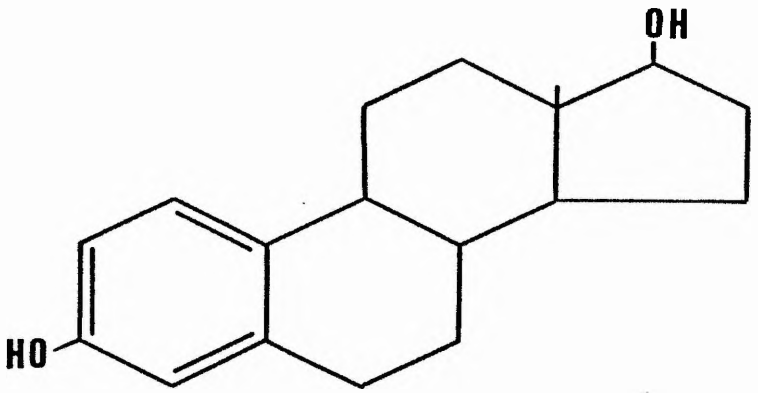


Fig. 2.10. Estradiol-17β (MW 272.4)

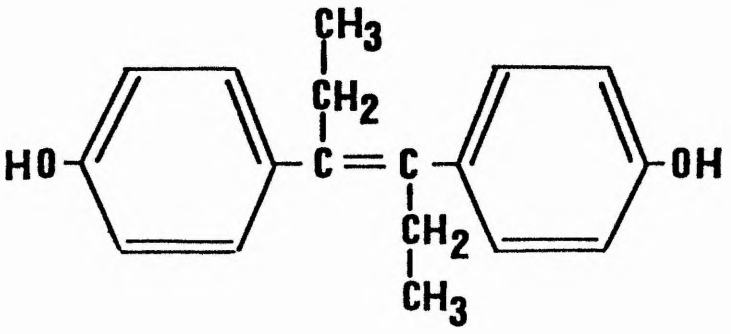


Fig. 2.11. Diethylstilboestrol (MW 268.3)

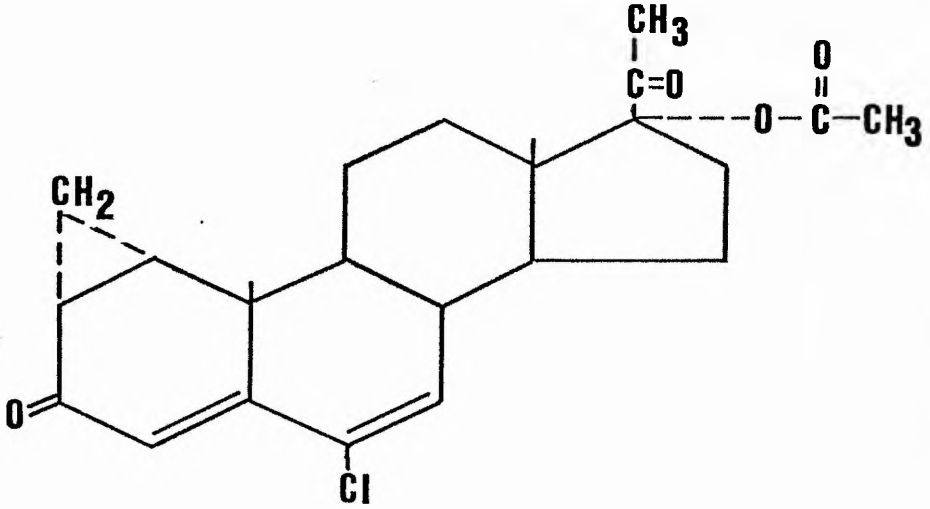


Fig. 2.12. Cyproterone acetate (MW 416.9)

2) Diethylstilboestrol Fig. 2.11 Sigma D-4628

2.1.6 Antiandrogens

Cyproterone acetate M20 (Fig. 2. 12) was kindly supplied by Schering AG Berlin/Bergkamen, West Germany. It was prepared as a 1 x 10^{-2}M solution in absolute ethanol and diluted such that the addition of 10μl to 3ml of culture medium produced the desired final concentrations. Control cultures received an equal volume of absolute ethanol.

2.1.7 ^{125}I-UdR Labelling Technique

5-^{125}Iodo-2'-deoxyuridine (^{125}I-UdR; Specific activity 5Ci mg^{-1}) was obtained from the Radiochemical Centre, Amersham, England. The isotope was diluted to a concentration of 10μCi ml^{-1} in RPMI 1640 culture medium supplemented with 50μg ml^{-1} kanamycin and 40μg ml^{-1} gentamicin. Each culture received 1μCi of ^{125}I-UdR per 3ml of Waymouth's medium, and unless otherwise stated, the labelling period was 24h. The explants were fixed in alcoholic Bouin's fluid, removed from the Millipore filters and washed in three changes of 70% alcohol during the next three days to remove any unbound isotope (Riches et al., 1976a). Incorporation of ^{125}I-UdR was determined using as LKB Wallac 1275 MiniGamma Counter. Groups of four explants, immersed in 70% alcohol, were counted for 10 minutes and the data adjusted for background radiation and decay of the isotope. The explants were then blotted on filter paper and weighed on a Sartorius 2434 balance. Results were expressed as counts per minute per milligram (CPM mg^{-1}) of fixed tissue.

2.1.8 DNA Extraction

Isolation of DNA (deoxyribonucleic acid) from ^{125}I-UdR labelled explants was performed using the method of Bonting and Jones (1957). Explants were transferred to ice cold 5% TCA (trichloroacetic acid) and the Millipore filters removed. The tissue was washed in three changes of cold 5% TCA during a 60 minute period, then rinsed twice in 0.15M saline. The wet weight of the tissue (2 explants per group) was determined using a Sartorius 2434 balance. Explants used for the extraction of DNA were homogenized in 1N NaOH for 60 minutes at 37°C. DNA was precipitated using 10% TCA and centrifugation at 1500rpm for 10 minutes. The precipitate was washed with 5% TCA and counted for 20 minutes in a IN Intertechnique CG30 Gamma Counter. Replicate explants were fixed in Bouin's fluid and counted as whole explants. The results were expressed as counts per minute per milligram (CPM mg^{-1}) of wet tissue.

2.1.9 Histology and Histoquantitation

Histological sections were prepared of the explants and biopsy specimens of fresh tissue (control before explantation). Fixed tissue was dehydrated in an ascending series of alcohol (75-100%), cleared in chloroform and embedded in paraplast. Sections were cut on a rotary microtome at 6μm and were stained using Erhlich's haematoxylin and 0.5% aqueous eosin, or the periodic-acid Schiff method with Mayer's haemalum as a nuclear counterstain (Pearse, 1968).

Histoquantitative measurements of epithelial height were determined using a Reichert-Jung Microstar 120 light microscope equipped with a Reichert-Jung MOP-1 console. Sections were examined using the X40 objective and epithelial

height (μm) was measured at 5 random sites in 30 alveoli. The results have been expressed as the mean height ± s.e.m. (standard error of the mean).

2.1.10 ^{3}H-TdR Labelling and Autoradiography

[methyl-^{3}H]-thymidine (^{3}H-TdR; Specific activity 5Ci mmole^{-1}) was obtained from the Radiochemical Centre, Amersham, England. The isotope was diluted to a concentration of 20μCi ml^{-1} in RPMI 1640 culture medium supplemented with 50μg ml^{-1} kanamycin and 40μg ml^{-1} gentamicin. Each culture received 1μCi of ^{3}H-TdR per ml of culture medium for a labelling period of 18h. The explants were fixed in Bouin's fluid and subsequently washed in three changes of 70% alcohol. Histological sections were prepared using the previously described (section 2.1.9) method of dehydration, embedding and sectioning.

Autoradiographs were prepared using the liquid emulsion coating technique described by Kopriwa and Leblond (1962). The slides were coated with Ilford Nuclear Research G5 emulsion and air dried overnight. As a control measure of background radiation, clean glass slides were prepared as autoradiographs. The slides were stored in light-proof boxes, containing silica gel, for 7-10 days at 4°C. Autoradiographs were developed using Kodak D19b High Contrast Developer (20°C) for 5 minutes, then rinsed in distilled water and fixed in Kodak Acid Fixer. Sections were post-stained with Mayer's haemalum and 0.5% aqueous eosin.

Kodak D19b High Contrast Developer was prepared as follows:

Sodium carbonate (anhydrous)	24.0g

Sodium sulphite (hydrous)	72.0g
Hydroquinone (Quinol)	4.4g
Potassium bromide	2.0g
Metol	1.1g
Distilled water	500ml

2.1.11 Statistical Methods

^{125}I-UdR Data

In each experiment, 12 or 16 explants were randomly allocated per treatment group and comprised three or four replicate cultures. Graphical presentation of the data represents results from replicate ($n \geq 3$) experiments calculated as treatment means±s.e.m. Variation among the treatments was determined using a two-way analysis of variance (with replication) and differences between individual treatment groups were determined using Duncan's Multiple Range comparison of means test.

2.2 HISTOCHEMICAL LOCALIZATION OF ACID PHOSPHATASE ACTIVITY

2.2.1 Tissue

Fresh specimens of ventral prostate were removed from young adult (4-6 months old) and retired breeding (>12 months old) rats derived from the Wistar strain. Biopsies of liver and kidney were also excised from young adult rats. Human benign prostatic hyperplasia (BPH) specimens obtained by transurethral resection were kindly supplied by Mr. J.P.A. Weaver, Consultant Urologist, Dundee Royal Infirmary.

2.2.2 Acid Phosphatase Staining of Cryostat Sections

Tissue specimens were quenched in a CO_2 expansion freezer and transferred to a Slee Type HRM cryostat (-20°C). Sections were cut at 8μm and mounted on glass coverslips. The method used for the histochemical demonstration of acid phosphatse activity was similar to the procedure described by Carson (1973). Replicate cryostat sections were fixed in either cold acetone (15 minutes) or 10% neutral formalin (5h and 24h) or were air dried without fixation. Sections were stained in the substrate (Napthol AS-MX phosphate; Sigma Chemical Company Ltd.) and dye (Fast Garnet GBC diazonium salt; Sigma Chemical Company Ltd.) solution for 60 minutes at room temperature. Mayer's haemalum was used as a nuclear counterstain and the coverslips were mounted onto glass slides with glycerine jelly. Control sections were incubated in either substrate or dye alone. Acid phosphatase activity was determined by the presence of a red, granular pigment.

2.3 CELL CULTURE

2.3.1 Animals

Male adult Wistar-derived rats age 2-3 months and weighing 200-250g were used as tissue donors. The animals were housed as described in section 2.1.1.

2.3.2. Tissue Disaggregation

The method used for the disaggregation of rat ventral prostate was similar to the technique described by Rubenstein

and Anderson (1980). Ventral prostate glands were aseptically removed from three animals by ether anaesthesia. The tissue was rinsed and decapsulated in pre-warmed (37°C) HEPES-buffered RPMI 1640 culture medium supplemented with 50IU ml^{-1} benzylpenicillin and 50μg ml^{-1} streptomycin sulphate (Flow Laboratories Ltd.). The tissue was pooled and transferred to 6ml of RPMI 1640 medium (37°C) containing 2mM L-glutamine, 50IU ml^{-1} benzylpenicillin, 50μg ml^{-1} streptomycin sulphate, 20% calf serum (Flow Laboratories Ltd.) and 0.1% collagenase (Clostridium histolyticum; Sigma Chemical Company Ltd.). The tissue was rapidly minced with scissors and then incubated at 37°C for 60 minutes. During the incubation period the tissue was mechanically disrupted by frequent pipetting into a 1ml syringe and in the last 5 minutes, 0.1ml of 0.4% DNase (Deoxyribonuclease I; Sigma Chemical Company Ltd.) was added (Clark et al., 1982). The homogenate was filtered through a nylon sieve (20μm; Begg Cousland Ltd.) and the cells were harvested in a bench centrifuge at 1000rpm for 10 minutes. The cells were washed in cold (4°C) RPMI 1640 culture medium containing 2mM L-glutamine, 50IU ml^{-1} benzylpenicillin, 50μg ml^{-1} streptomycin sulphate and 20% calf serum (hereafter referred to as "medium"). The cells were then harvested in an MSE Chilspin refrigerated centrifuge (Fisons Ltd.) for 10 minutes at 1000rpm and resuspended in 1ml of cold medium.

2.3.3 Isolation of Rat Prostatic Epithelial Cells

Discontinuous density gradients were prepared by layering Percoll^{R} (Pharmacia Fine Chemicals AB) solutions of the following densities into a 10ml conical centrifuge tube (Sterilin Ltd.):

$1.02\ g\ ml^{-1}$ TOP

$1.04\ g\ ml^{-1}$

$1.06\ g\ ml^{-1}$

$1.08\ g\ ml^{-1}$

$1.10\ g\ ml^{-1}$ CUSHION

Percoll solutions of the appropriate densities were prepared using either 1.5M saline or 10X concentrated RPMI 1640 culture medium (Flow Laboratories Ltd.) such that the individual bands of the gradient were alternately coloured and easily distinguished. The following formula (Percoll^R Methodology and Applications, 1980) was used to prepare the necessary solutions:

$$V_0 = V \frac{\rho - 0.1\rho_{10} - 0.9}{\rho_0 - 1}$$

where V_0 = volume of Percoll (undiluted) ml

V = volume of the final solution ml

ρ = desired density of the final solution $g\ ml^{-1}$

ρ_0 = density of undiluted Percoll (1.133) $g\ ml^{-1}$

ρ_{10} = density of 1.5M saline (1.055) $g\ ml^{-1}$

or density of 10X RPMI (1.057) $g\ ml^{-1}$

Since the refractive index of Percoll is directly proportional to density, an Abbe '60' refractometer (Bellingham Stanley Ltd.) was used to verify the densities of the prepared solutions.

The cells obtained from the tissue disaggregation, suspended in 1ml of medium, were gently layered onto the top of the gradient and then separated by isopycnic centrifugation in the Chilspin centrifuge for 25 minutes at 1500rpm. Using a Pasteur pipette, the individual cell bands were serially removed from the top of the gradient and then washed three times in cold medium to remove any Percoll.

2.3.4 Characterization of Isolated Rat Prostatic Epithelial Cells

Cytological examination of each cell fraction was performed using cytocentrifuge (Shandon Southern Instruments Ltd.) preparations. Aliquots (0.2ml) of each cell fraction were centrifuged onto glass slides for 5 minutes at 400rpm. The slides were air dried, fixed in methanol and stained using a modified Jenner-Giemsa technique (Thomas, 1971). Morphological assessment of the cells indicated that the fraction removed from the 1.04 g ml^{-1} interface contained mainly epithelial cells.

The histochemical demonstration of acid phosphatase was used to identify cells of epithelial origin. Replicate cytocentrifuge preparations of the epithelial-enriched fraction were fixed in cold acetone or 10% neutral formalin for 1 minute or were air dried without fixation, and were then stained for acid phosphatase (as described in section 2.2.2).

Epithelial cell viability was determined using the trypan blue dye exclusion method. An aliquot of the cell suspension was diluted 4:1 with trypan blue (0.2%) and the fraction of the cells excluding the dye were counted using an improved Neubaur haemocytometer.

2.3.5 Cell Culture and Labelling (^{125}I-UdR; ^{3}H-TdR) Methods

Similar to Rubenstein and Anderson (1980), cells derived from the epithelial-enriched fraction were cultured in HEPES-buffered RPMI 1640 medium supplemented with 2mM L-glutamine, 50IU ml^{-1} benzylpenicillin, 50μg ml^{-1} streptomycin sulphate, 3μg ml^{-1} bovine insulin, 10% calf serum and 4×10^{-7}M testosterone.

Short term monolayer cell cultures were prepared by plating 10^{6} cells in 5ml of medium into 50ml plastic tissue

culture flasks (Sterilin Ltd.). The cultures were then incubated at 37°C in a humidified air and 5% CO_2 incubator. After one week the cells were stained using either the Jenner-Giemsa technique (as described in section 2.3.4) or the acid phosphatase staining technique (as described in section 2.2.2).

^{125}I-UdR Labelling

"Nuclon" microtest plates (96 flat bottom wells; Gibco Europe Ltd.) were inoculated with 5 x 10^4 cells per well and incubated at 37°C in a humidified air and 5% CO_2 incubator for 9 days. Every second day six replicate wells were labelled with 0.1μCi of ^{125}I-UdR for 24h. The cells were then trypsinized (2.5% trypsin; Sigma Chemical Company Ltd.) and collected onto fibre filter discs using a Titertek (Skatron AS) cell harvester. The filter discs were dried and counted in the LKB Wallac 1275 MiniGamma Counter for 5 minutes to determine ^{125}I-UdR uptake. The results have been expressed as counts per minute (CPM).

^{3}H-TdR Labelling

Coverslip cultures were prepared using "Multidish" culture plates (24 wells; Flow Laboratories Ltd.) lined with round, plastic Thermanox coverslips (13mm; Lux Scientific Corporation). Each plate was inoculated with 10^5 cells per well and incubated in a humidified air and 5% CO_2 incubator at 37°C for 9 days. Every second day, four replicate cultures were labelled with 0.2μCi of ^{3}H-TdR for 18h and then fixed in acetic ethanol. The coverslips were mounted onto glass slides with glycerine jelly and prepared as autoradiographs using the liquid emulsion coating technique (as described in section 2.1.10). The slides were post-stained with Mayer's haemalum

and 0.5% aqueous eosin. Replicate cultures were stained for acid phosphatase activity (as described in section 2.2.2).

CHAPTER 3

EXPERIMENTAL PROCEDURE AND RESULTS

3.1 PROLIFERATIVE RESPONSES OF RAT VENTRAL PROSTATE IN ORGAN CULTURE

The proliferative activity of rat ventral prostate in organ culture was monitored using the incorporation of ^{125}I-UdR as a measure of DNA synthesis. ^{125}I-UdR uptake has been expressed as:

$$\frac{^{125}\text{I-UdR uptake of treatment group (CPM mg}^{-1})}{^{125}\text{I-UdR uptake of non-supplemented control (CPM mg}^{-1})} \times 100$$

such that the incorporation of ^{125}I-UdR by the unsupplemented control equals 100 CPM mg^{-1}.

Results are presented as graphs and photomicrographs which are located at the end of section 3.1.

3.1.1 Proliferative Responses to Testosterone in Young Adult Rat Ventral Prostate

^{125}I-UdR Results

The proliferative activity of young adult (4-6 months old) rat ventral prostate cultured in chemically-defined medium, in the absence and presence of testosterone (4×10^{-9} to 4×10^{-5}M), during a four day culture period is shown in Fig. 3.1. Testosterone supplements were added on day 0 and replenished following the medium change on day 2. Replicate cultures were labelled each day with ^{125}I-UdR for 24h. Cultures treated with 4×10^{-9}, 4×10^{-8}, 4×10^{-7} and 4×10^{-6}M testosterone showed similar stimulation of ^{125}I-UdR uptake with time, whereas 4×10^{-5}M testosterone was markedly inhibitory. ^{125}I-UdR uptake in testosterone-free control cultures gradually decreased with time.

Using the same procedure and a six day culture period,

Fig. 3.2 illustrates the pattern of the proliferative response to 4×10^{-7}M testosterone. ^{125}I-UdR uptake in testosterone-stimulated cultures reached peak activity on day 4 and declined thereafter. The incorporation of ^{125}I-UdR in testosterone-free control cultures remained relatively constant throughout the culture period.

As shown in Fig. 3.3, ^{125}I-UdR uptake in cultures treated with 4×10^{-7}M testosterone increased linearly during the final 24h of a four day culture period. ^{125}I-UdR was added at the onset of day 4 and replicate cultures were harvested at four hourly intervals. By comparison with the testosterone treated cultures, ^{125}I-UdR uptake in the control cultures showed only a slight increase during day 4.

The relationship between testosterone concentration and ^{125}I-UdR uptake on day 4 is shown in Fig. 3.4. During the four day culture period, testosterone was added on day 0 and renewed on day 2. Testosterone concentrations ranging from 4×10^{-9} to 4×10^{-6}M showed similar stimulation of ^{125}I-UdR uptake, with maximal incorporation occurring at 4×10^{-7} and 4×10^{-6}M. Concentrations of testosterone less than 4×10^{-10}M were not stimulatory and 4×10^{-5}M was inhibitory.

Using the same procedure, the inhibitory effect of high concentrations of testosterone was investigated further (Fig. 3.5). Optimal stimulation of ^{125}I-UdR uptake, as shown in Fig. 3.4, was maintained in cultures treated with 0.5×10^{-5}, 1.5×10^{-5} and 2.0×10^{-5}M testosterone. The onset of the inhibitory response occurred at 2.5×10^{-5}M testosterone.

The relationship between ^{125}I-UdR uptake on day 4 and testosterone concentration was compared in whole explants and DNA extracted from replicate cultures (Fig. 3.6). Irrespective of testosterone concentration, ^{125}I-UdR uptake was equivalent in whole explants and corresponding DNA precipitates.

The temporal requirement for testosterone (4×10^{-7}M) in relation to maximum ^{125}I-UdR uptake on day 4 is shown in Fig. 3.7. Testosterone was available for the following time intervals during the four day culture period: (1) the first 24h, (2) the second 24h, (3) the first 48h, (4) the final 48h or (5) the entire 96h. After treatment with testosterone, the explants were rinsed in Waymouth's medium and transferred to new grids and Petri dishes containing testosterone-free medium. Regardless of the period of availability, ^{125}I-UdR uptake was maximal when testosterone was present during the first 48h of the culture period. The addition of testosterone after this time reduced the magnitude of the stimulatory response on day 4.

The effect of delaying the introduction of testosterone on the pattern of the proliferative response is shown in Fig. 3.8. Cultures were incubated with 4×10^{-7}M testosterone for either the entire six day culture period (by addition on day 0, 2 and 4) or the final 96h (by addition on day 2 and 4). Each day replicate cultures were labelled with ^{125}I-UdR for 24h. Despite the delay in the introduction of testosterone, peak activity still occurred on day 4, but the magnitude of the response was markedly reduced.

Using a four day culture period, the effects of

5% foetal calf serum and/or insulin (3μg ml^{-1}) on the incorporation of ^{125}I-UdR were investigated in cultures maintained in the absence and presence of testosterone (4×10^{-7} and 4×10^{-5}M) (Fig. 3.9). Testosterone was added on day 0 and replenished following the medium change on day 2. Treatment with 4×10^{-7}M testosterone produced maximal ^{125}I-UdR uptake under all conditions, whereas 4×10^{-5}M testosterone was consistently least effective. The presence of foetal calf serum did not significantly ($p>0.05$) alter the responses observed in the serum-free system. However, the presence of insulin stimulated an increase in the uptake of ^{125}I-UdR by the testosterone-free control cultures and enhanced the stimulatory effect of 4×10^{-7}M testosterone. The combination of foetal calf serum and insulin stimulated increases of similar magnitude in the ^{125}I-UdR uptake of all cultures.

In order to determine optimal conditions for ^{125}I-UdR uptake, explants were grown on Millipore filter paper mounted on the grid, directly on the grid or submerged in chemically-defined medium (Fig. 3.10). During the four day culture period, testosterone (4×10^{-7}M) supplements were added on day 0 and 2. All cultures were submerged and labelled with ^{125}I-UdR for the final 24h. Regardless of the experimental conditions, all cultures maintained in the presence of testosterone exhibited a similar stimulatory response on day 4. ^{125}I-UdR uptake in control cultures which had been submerged for four days was similar to that of testosterone treated cultures and was significantly ($p < 0.01$) greater than the paper or grid controls.

Histological Observations

The *in vivo* histological appearance of young adult rat ventral prostate, as shown in a fresh biopsy specimen (Plate 3.1), consists of glandular acini and ducts separated by thin strands of fibromuscular stroma. A single layer of straight or folded secretory epithelium lines the alveoli and ducts, and is composed of cuboidal or columnar epithelial cells that have a round basal nucleus, with a conspicuous nucleolus, and exhibit a distinctive supranuclear clear zone. Eosinophilic or PAS-positive secretory material is often observed as a thin film in the alveolar lumina and at the apical surface of the epithelial cells. Occasionally, small round basal cells are seen wedged between the secretory epithelium and the basement membrane.

Ventral prostatic tissue cultured for four days in non-supplemented chemically-defined medium underwent epithelial atrophy (Plate 3.2). The histological architecture of the tissue was maintained but most alveoli were dilated and lined with flattened epithelial cells. Residual secretory material was present in some alveolar lumina, but evidence of active secretion was usually absent. Alveolar epithelium located at the peripheral edges of the explants often exhibited regions of epithelial hyperplasia, which appeared as small epithelial outgrowths or a piling up of cells into the lumen. In these alveoli, the lumina usually contained extruded epithelial cells and cellular debris. The content of fibromuscular stroma appeared to vary between explants, but never deviated markedly from that seen in the *in vivo* controls.

As shown in Plate 3.3, supplementation of the culture medium with 4×10^{-12}M testosterone did not prevent the occurrence of epithelial atrophy observed in the non-supplemented controls. Most alveoli were lined with flattened epithelial cells and secretory activity was diminished or absent. Similar results were observed in cultures treated with 4×10^{-11} and 4×10^{-10}M testosterone. However, cultures treated with 4×10^{-9}, 4×10^{-8}, 4×10^{-7} or 4×10^{-6}M testosterone were histologically similar to the in vivo controls in that epithelial height and secretory activity were maintained. Varying degrees of epithelial hyperplasia were also observed, but was most prominent at the higher testosterone concentrations. In cultures treated with 4×10^{-9}M testosterone (Plate 3.4), the secretory epithelium of centrally located alveoli was well-maintained, while peripheral alveoli usually exhibited some degree of epithelial hyperplasia. Treatment with 4×10^{-7}M testosterone appeared to promote epithelial cell proliferation throughout the explant (Plate 3.5). Regions of well-maintained and hyperplastic epithelium often occurred within the same alveolus, but in the hyperplastic areas, epithelial height and secretory activity were not usually maintained and the lumen often contained extruded cells. Changes in the appearance of the epithelium in relation to testosterone treatment were also reflected in measurements of epithelial height (Table 2.1).

Treatment with 4×10^{-5}M testosterone appeared to have a cytotoxic effect on the tissue (Plate 3.6). In the majority of explants the epithelium was necrotic and

Table 2.1 Average epithelial height (μm ± s.e.m.) of young adult rat ventral prostate *in vivo* and *in vitro*.

BIOPSY	4 - DAY ORGAN CULTURE			
	Control	Testosterone		
		$4x10^{-12}$M	$4x10^{-9}$M	$4x10^{-7}$M
17.12± 0.42	9.01± 0.31	10.54± 0.47	15.35± 0.50	16.85± 0.61

the lumina were occulded with cellular debris. Fibromuscular tissue was scarce and often appeared as only a few isolated fibroblasts.

Autoradiographic localization of ^{3}H-TdR incorporation correlated well with the histological appearance of ^{125}I-UdR labelled explants. In four day cultures maintained in non-supplemented medium, ^{3}H-TdR labelling was primarily associated with epithelial outgrowths at the periphery of the explants (Plate 3.7). However, in cultures treated with 4×10^{-7}M testosterone, ^{3}H-TdR labelling was homogeneously distributed throughout the tissue and was predominantly associated with the basal regions of the alveolar epithelium (Plate 3.8). Cultures treated with 4×10^{-5}M testosterone were almost entirely necrotic and showed no evidence of ^{3}H-TdR labelling.

Cultures maintained in testosterone-free medium supplemented with either 5% foetal calf serum or insulin ($3\mu g\ ml^{-1}$) underwent epithelial atrophy similar to those explants grown in non-supplemented chemically-defined medium. However, the presence of insulin appeared to enhance the proliferative activity of epithelial cells located at the periphery of the explants. Cultures maintained in the presence of both insulin and serum exhibited similar peripheral epithelial hyperplasia, but the remainder of the tissue appeared well-maintained and resembled cultures which had been treated with optimal concentrations of testosterone.

The histological appearance of cultures treated with 4×10^{-7}M testosterone were similar whether the explants were grown in chemically-defined medium or media supplemented with insulin and/or foetal calf

serum. Most alveoli exhibited regions of well-preserved secretory epithelium and areas of hyperplastic growth.

Treatment with 4×10^{-5}M testosterone remained cytotoxic in the presence of insulin, but in media containing foetal calf serum the severity of the cytotoxic response was marginally reduced as some alveoli were partially maintained. However, in the presence of insulin, foetal calf serum and 4×10^{-5}M testosterone most explants were well-maintained and indistinguishable from those which had been treated with optimal concentrations of testosterone.

Cultures maintained in chemically-defined medium either on Millipore filter paper or directly on the grid were histologically similar. Those cultured in the absence of testosterone underwent epithelial retrogression, whereas treatment with 4×10^{-7}M testosterone maintained the secretory epithelium and promoted epithelial cell proliferation. In contrast, the secretory epithelium in explants cultured by submersion was sloughed into the lumen, often in long continuous segments, irrespective of the presence or absence of testosterone. The extruded epithelium was replaced by two to three irregular layers of crowded, round basal cells. With the exception of the perialveolar stroma, the interacinar tissue in these cultures often appeared hypocellular.

3.1.2 Proliferative Responses to Testosterone and its Metabolites in Young Adult Rat Ventral Prostate

Using the ^{125}I-UdR labelling technique, the proliferative response of cultured rat ventral prostate

to testosterone stimulation was compared with that of its major metabolites. Explants were cultured on Millipore filter paper supported on stainless steel grids in chemically-defined medium and were treated with testosterone, 5α-dihydrotestosterone, androstanedione, androstenedione, androstane-3β,17β-diol or 5β-dihydrotestosterone at concentrations of 4×10^{-9}, 4×10^{-7} or 4×10^{-5}M. Androgen supplements were added on day 0 and replenished on day 2 following the medium change. Cultures were labelled with ^{125}I-UdR for the final 24h of a four day culture period.

^{125}I-UdR Results

The proliferative responses to testosterone and 5α-dihydrotestosterone (Fig. 3.11) were not significantly different ($p > 0.05$). Concentrations of 4×10^{-9} and 4×10^{-7}M testosterone or 5α-dihydrotestosterone significantly stimulated ^{125}I-UdR uptake ($p < 0.01$), whereas at 4×10^{-5}M these androgens were both equally inhibitory. However, in contrast to 5α-dihydrotestosterone, its epimer 5β-dihydrotestosterone did not stimulate ^{125}I-UdR uptake at any of the concentrations used (Fig. 3.12).

As shown in Fig. 3.13, the proliferative responses to testosterone, androstanedione and androstenedione were all similar, whereas androstane-3β,17β-diol elicited a different pattern of response. Testosterone, androstanedione and androstenedione significantly ($p < 0.01$) stimulated ^{125}I-UdR uptake at concentrations of 4×10^{-9} and 4×10^{-7}M, and were all equally

inhibitory at 4×10^{-5}M. In contrast, 4×10^{-9}M androstane-3β,17β-diol did not stimulate the incorporation of ^{125}I-UdR, whereas 4×10^{-7} and 4×10^{-5}M were both marginally stimulatory.

Histological Observations

The histological appearance of cultures treated with 5α-dihydrotestosterone, androstanedione or androstenedione at concentrations of 4×10^{-9}, 4×10^{-7} and 4×10^{-5}M resembled cultures which had been treated with equimolar concentrations of testosterone. At 4×10^{-5}M all of these androgens were markedly cytotoxic, whereas at concentrations of 4×10^{-7} and 4×10^{-9}M the secretory epithelium was well-maintained and epithelial cell proliferation was stimulated (Plate 3.9). Unlike these androgens, 4×10^{-5}M androstane-3β,17β-diol was not cytotoxic (Plate 3.10). At concentrations of 4×10^{-5} and 4×10^{-7}M, androstane-3β,17β-diol fully maintained the height and secretory activity of the alveolar epithelium without promoting pronounced epithelial hyperplasia. At 4×10^{-9}M androstane-3β,17β-diol was inactive and epithelial atrophy occurred.

In contrast to 5α-dihydrotestosterone, treatment with 5β-dihydrotestosterone (Plate 3.11) was ineffective at all concentrations used and the cultures resembled those which had been maintained in androgen-free medium.

3.1.3 Proliferative Responses to Testosterone in Ventral Prostate from Retired Breeding Rats

Using the previously established method of quantitative organ culture (as described in section 3.1.2), the proliferative response to testosterone was monitored in ventral prostatic tissue from retired breeding rats (> 12 months old).

^{125}I-UdR Results

The relationship between testosterone concentration and ^{125}I-UdR uptake following four days of organ culture is shown in Fig. 3.14. Unlike the testosterone dose-response curve for young adult rat ventral prostate (Fig. 3.4), ^{125}I-UdR uptake in cultures of older prostatic tissue showed only a slight increase following treatment with 4×10^{-9}, 4×10^{-8} and 4×10^{-7}M testosterone, and both 4×10^{-6} and 4×10^{-5}M testosterone were inhibitory.

Using a six day culture period, Fig. 3.15 illustrates the pattern of the proliferative response to 4×10^{-7}M testosterone in aged rat ventral prostate. In contrast to young adult rat ventral prostate (Fig. 3.2), ^{125}I-UdR uptake in older prostatic tissue followed a similar pattern both in the presence and absence of testosterone. Maximum ^{125}I-UdR uptake occurred on day 4 to 5 and the testosterone stimulated response was not significantly different ($p > 0.05$) from that observed in the control.

Histological Observations

Macroscopically, ventral prostate from retired breeding rats appeared larger than that from younger

animals and explantation required greater force. As shown in Plate 3.12, the *in vivo* (control before explantation) histological appearance of aged rat ventral prostate was highly variable. Many alveoli were dilated and lined with flattened epithelial cells, while others exhibited typical folded columnar epithelium, but often both types of epithelium were present within a single alveolus. Secretory activity was absent in regions of atrophic epithelium and the lumina often contained small, eosinophilic concretions.

In contrast to young adult rat ventral prostate, aged tissue cultured either in the absence (Plate 3.13) or presence of 4×10^{-7}M testosterone (Plate 3.14) exhibited similar histology. In most explants degenerative changes were evident in the interacinar tissue, which included loss of the perialveolar stroma, as well as the basement membrane of the alveolar epithelium, resulting in the epithelium having an irregular appearance which was further distorted by regions of hyperplastic growth. Treatment with higher concentrations of testosterone (4×10^{-6} and 4×10^{-7}M) had a cytotoxic effect on both the epithelium and stroma, similar to that observed in younger tissue treated with 4×10^{-5}M testosterone.

Fig. 3.1 Incorporation of ^{125}I-UdR by explants of young adult rat ventral prostate cultured in chemically-defined medium for up to four days in the absence (Control) and presence of testosterone (4×10^{-9}, 4×10^{-8}, 4×10^{-7}, 4×10^{-6} and 4×10^{-5}M).

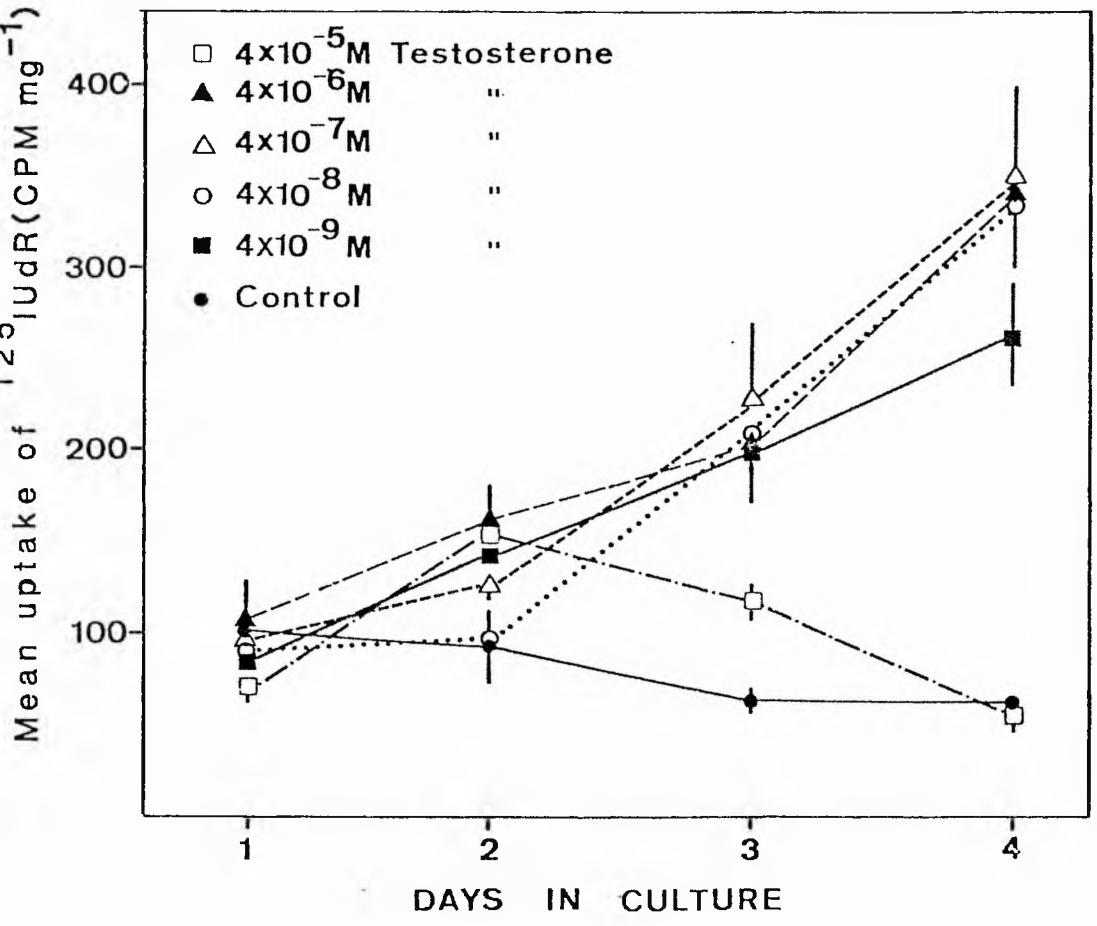

□ 4x10⁻⁵M Testosterone
▲ 4x10⁻⁶M "
△ 4x10⁻⁷M "
○ 4x10⁻⁸M "
■ 4x10⁻⁹M "
● Control
Mean uptake of 125IUdR(CPM mg⁻¹)
400
300
200
100
1
2
3
4
DAYS IN CULTURE

Fig. 3.2 Incorporation of ^{125}I-UdR by explants of young adult rat ventral prostate cultured in chemically-defined medium for up to six days in the absence (Control) and presence of testosterone (4×10^{-7}M).

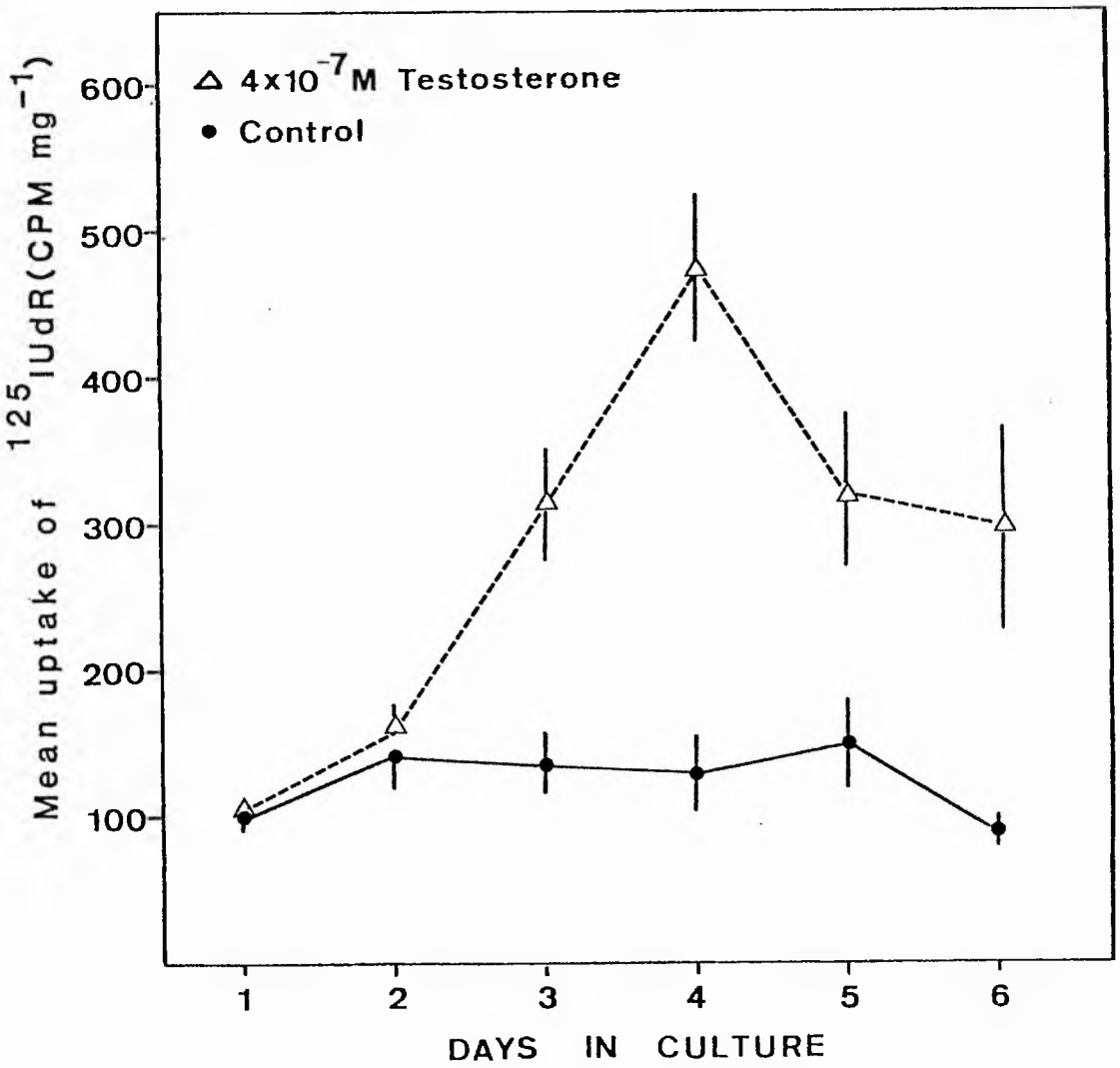

Δ 4x10⁻⁷M Testosterone
• Control
Mean uptake of ¹²⁵IUdR(CPM mg⁻¹)
600
500
400
300
200
100
1
2
3
4
5
6
DAYS IN CULTURE

Fig. 3.3 ^{125}I-UdR uptake during the final 24h of a four day culture period by explants of young adult rat ventral prostate maintained in chemically-defined medium in the absence (Control) and presence of testosterone (4×10^{-7}M).

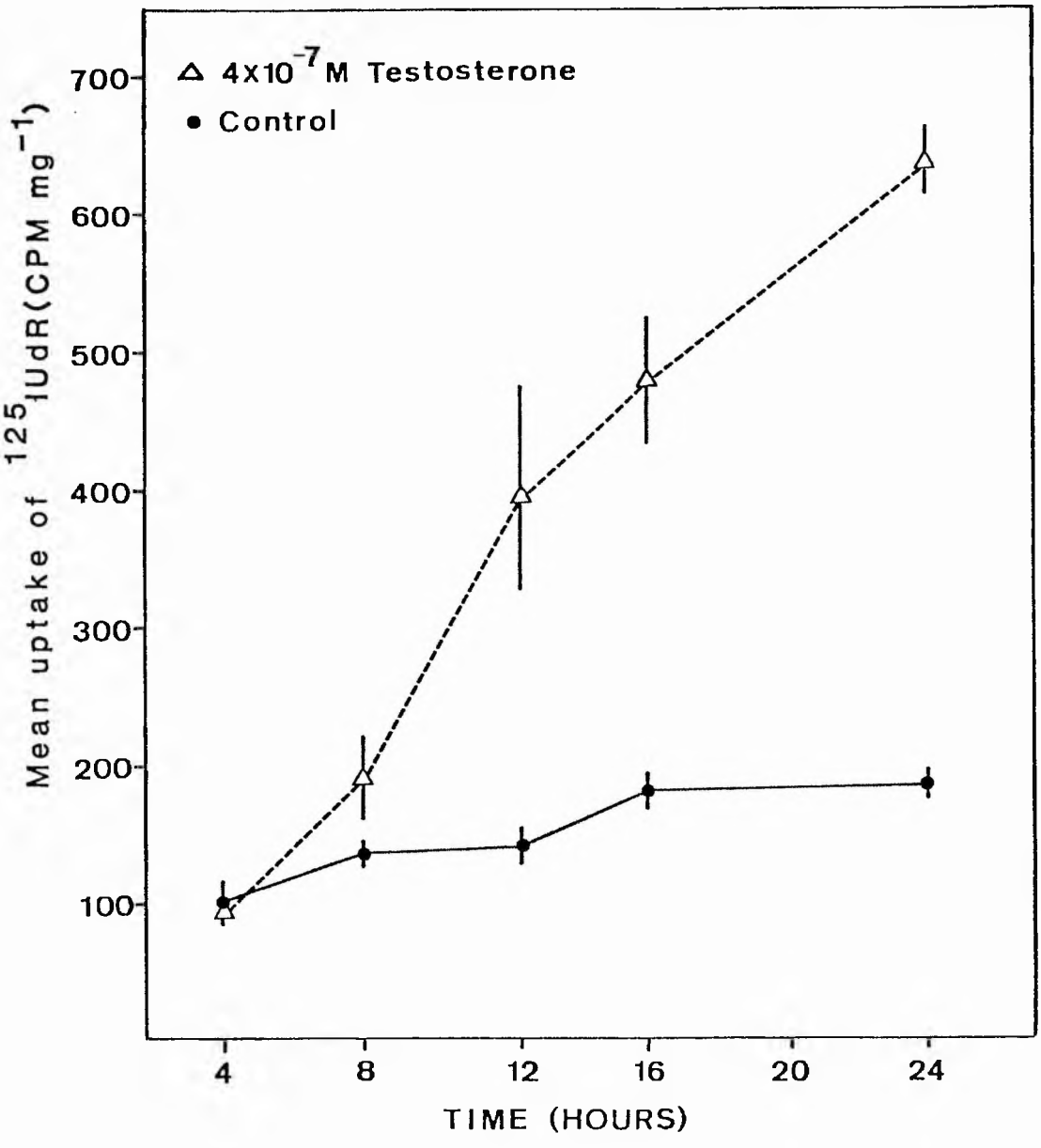

△ 4X10⁻⁷M Testosterone
● Control
Mean uptake of ¹²⁵IUdR(CPM mg⁻¹)
700
600
500
400
300
200
100
4
8
12
16
20
24
TIME (HOURS)

Fig. 3.4 Incorporation of ^{125}I-UdR by explants of young adult rat ventral prostate following four days of organ culture in chemically-defined medium in the absence (■, medium only; □, medium and alcohol diluent) and presence of testosterone (4×10^{-12}, 4×10^{-11}, 4×10^{-10}, 4×10^{-9}, 4×10^{-8}, 4×10^{-7}, 4×10^{-6} and 4×10^{-5}M).

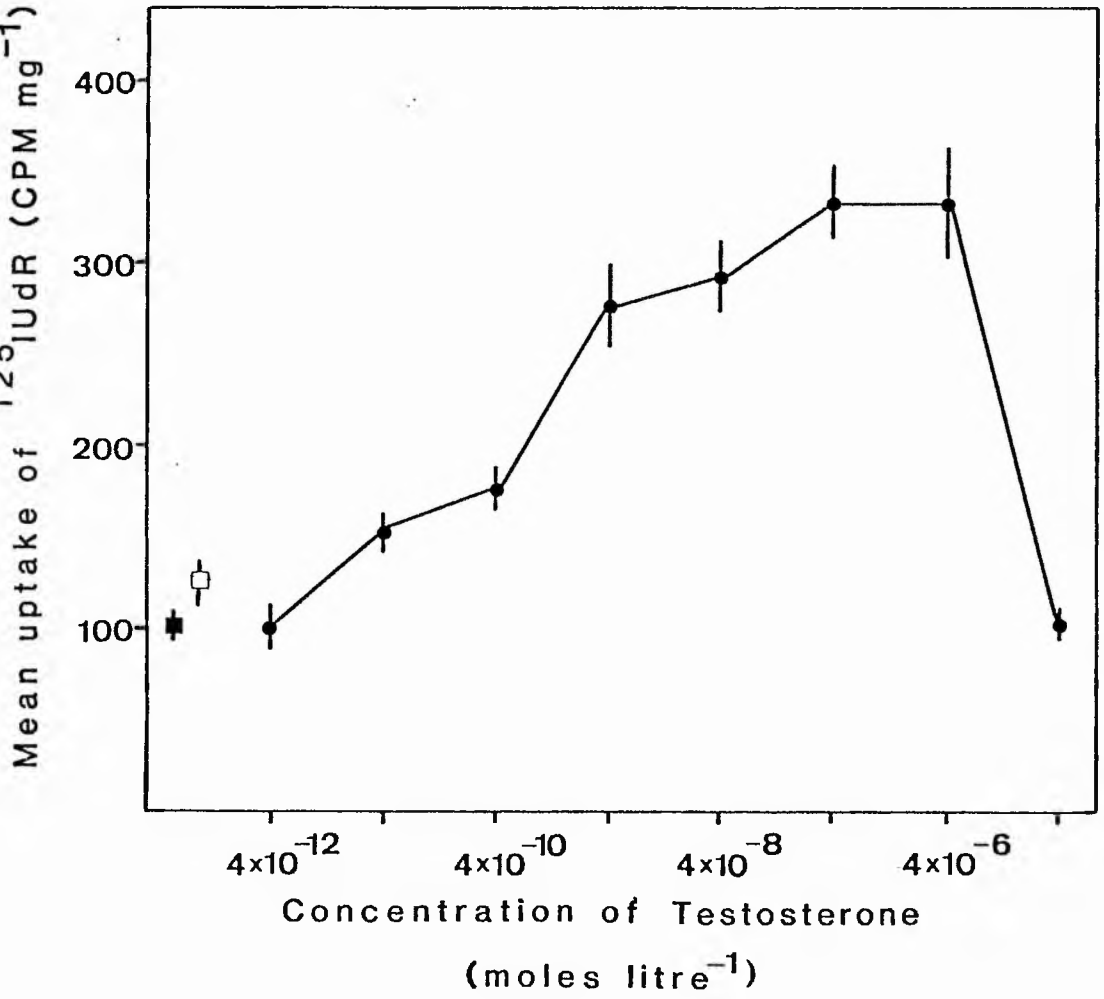

Mean uptake of ^{125}IUdR (CPM mg^{-1})
400
300
200
100
4x10^{-12}
4x10^{-10}
4x10^{-8}
4x10^{-6}
Concentration of Testosterone
(moles litre^{-1})

Fig. 3.5 Incorporation of ^{125}I-UdR by explants of young adult rat ventral prostate following four days of organ culture in chemically-defined medium in the absence (■, medium only; □, medium and alcohol diluent) and presence of testosterone (0.5×10^{-5}, 1.5×10^{-5}, 2.0×10^{-5}, 3.0×10^{-5}, 3.5×10^{-5} and 4×10^{-5}M).

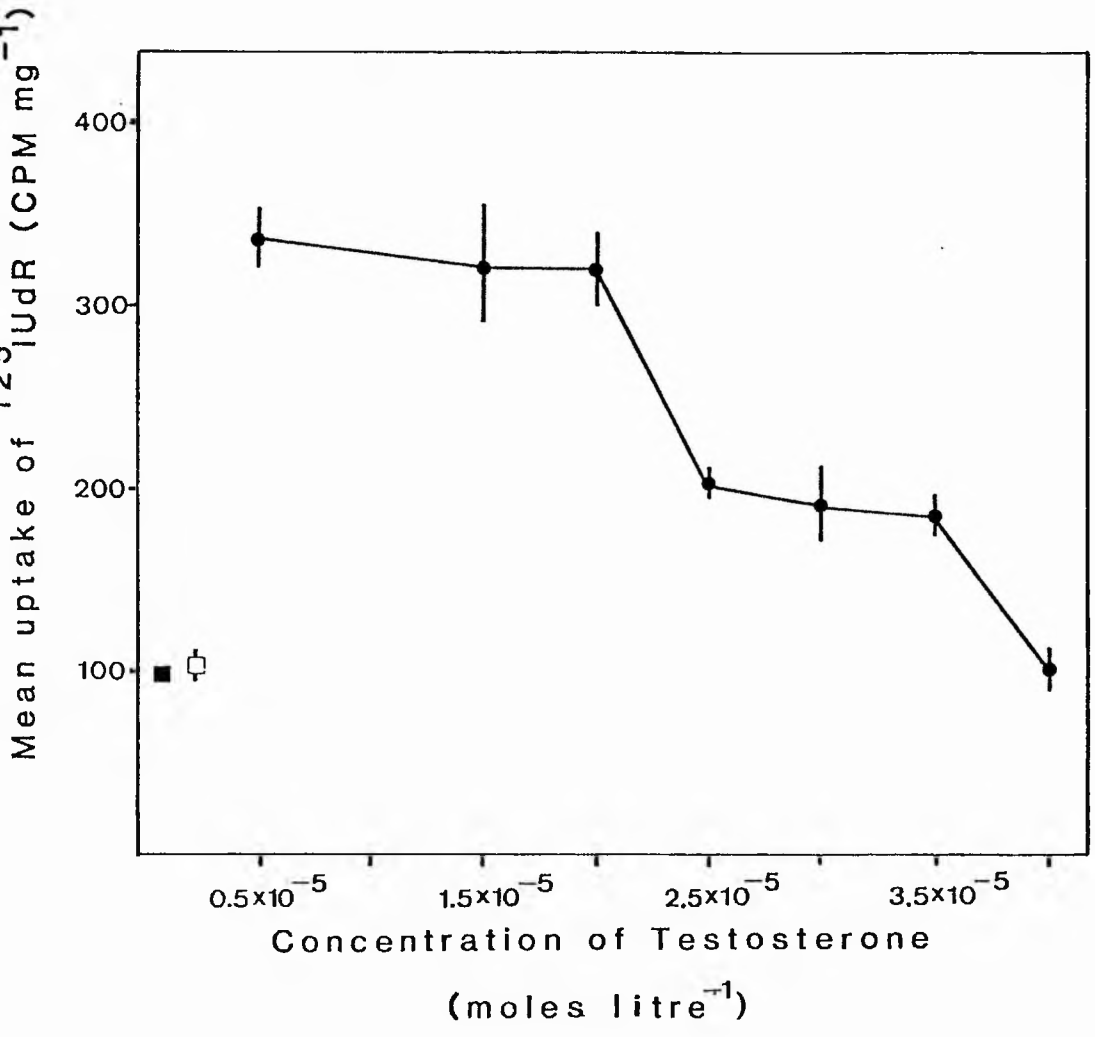

Mean uptake of ^{125}IUdR (CPM mg^{-1})
400
300
200
100
0.5x10^{-5}
1.5x10^{-5}
2.5x10^{-5}
3.5x10^{-5}
Concentration of Testosterone
(moles litre^{-1})

Fig. 3.6 Incorporation of ^{125}I-UdR in whole explants and DNA extracted from replicate cultures of young adult rat ventral prostate following four days of organ culture in chemically-defined medium in the absence and presence of 4×10^{-12}, 4×10^{-9}, 4×10^{-7} and 4×10^{-5}M testosterone (T).

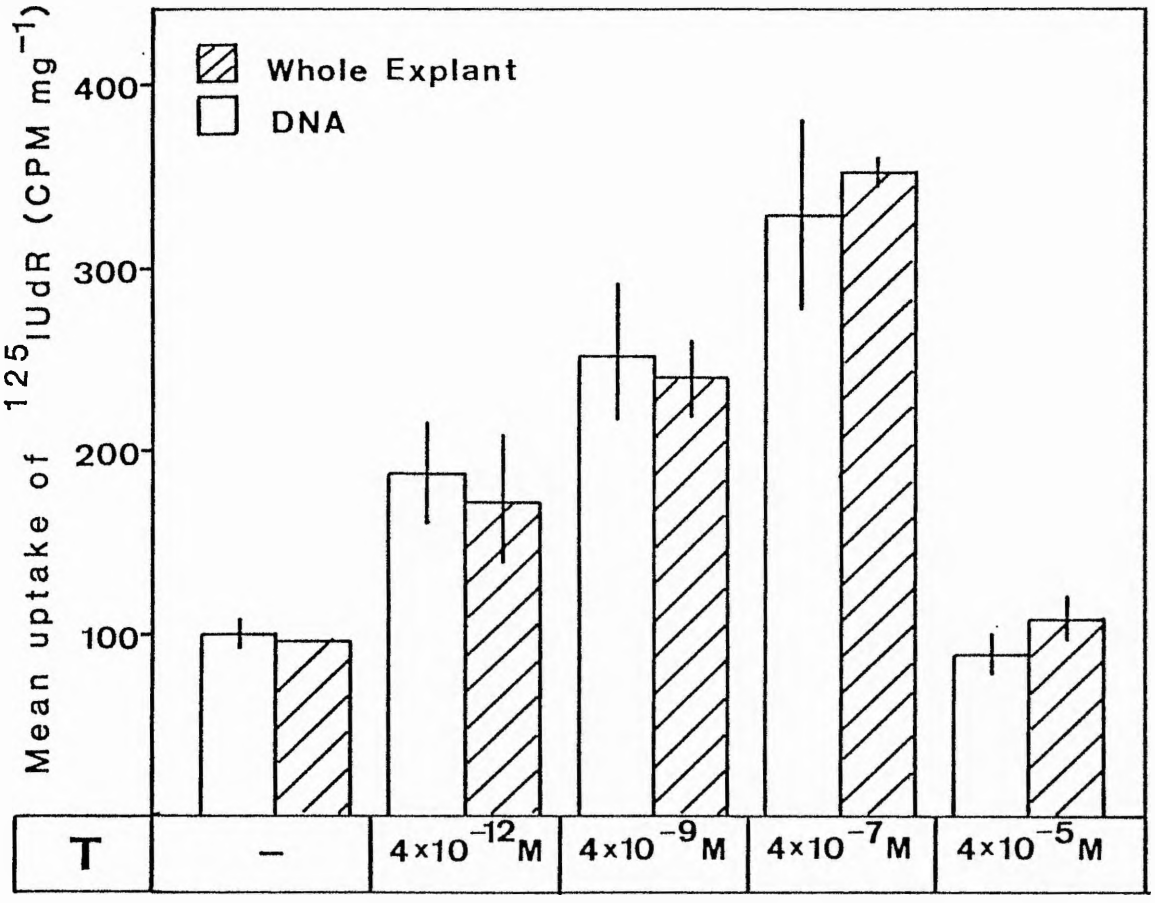

Whole Explant
DNA
Mean uptake of ^{125}IUdR (CPM mg^{-1})
400
300
200
100
T
–
4×10^{-12}M
4×10^{-9}M
4×10^{-7}M
4×10^{-5}M

Fig. 3.7 Incorporation of ^{125}I-UdR by explants of young adult rat ventral prostate following four days of organ culture in chemically-defined medium in the absence and presence of 4×10^{-7}M testosterone. 'TIME' represents the time interval during which testosterone was present in the medium. 'CONTROL' represents cultures which were maintained in medium only, or in medium supplemented with the alcohol diluent.

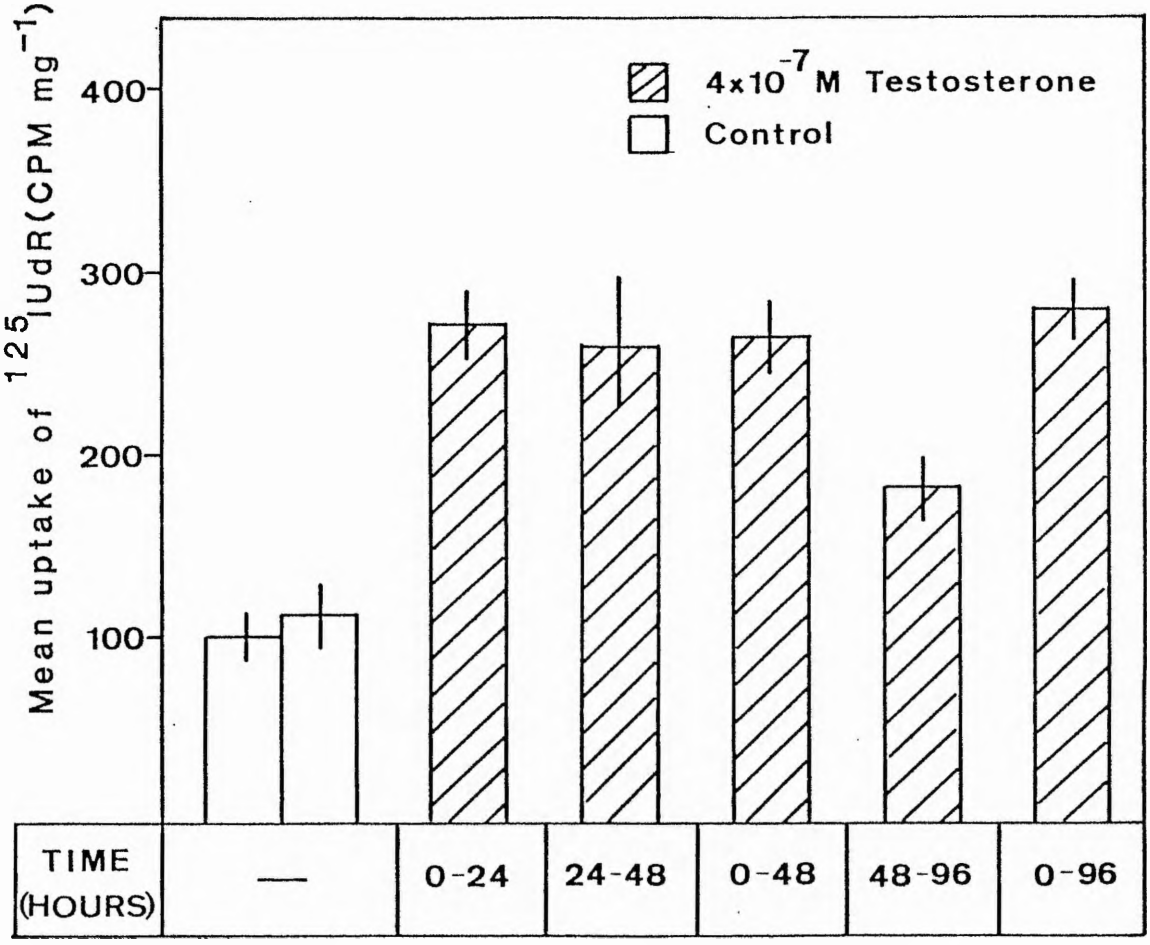

Mean uptake of ^{125}IUdR(CPM mg^{-1})
400
300
200
100
4x10^{-7}M Testosterone
Control
TIME (HOURS)
—
0-24
24-48
0-48
48-96
0-96

Fig. 3.8 Incorporation of ^{125}I-UdR by explants of young adult rat ventral prostate cultured in chemically-defined medium for up to six days in the absence and presence of testosterone. Testosterone was present for either the entire culture period (△), or only the final 96h (▲). 'Control' represents cultures that were maintained in testosterone-free medium.

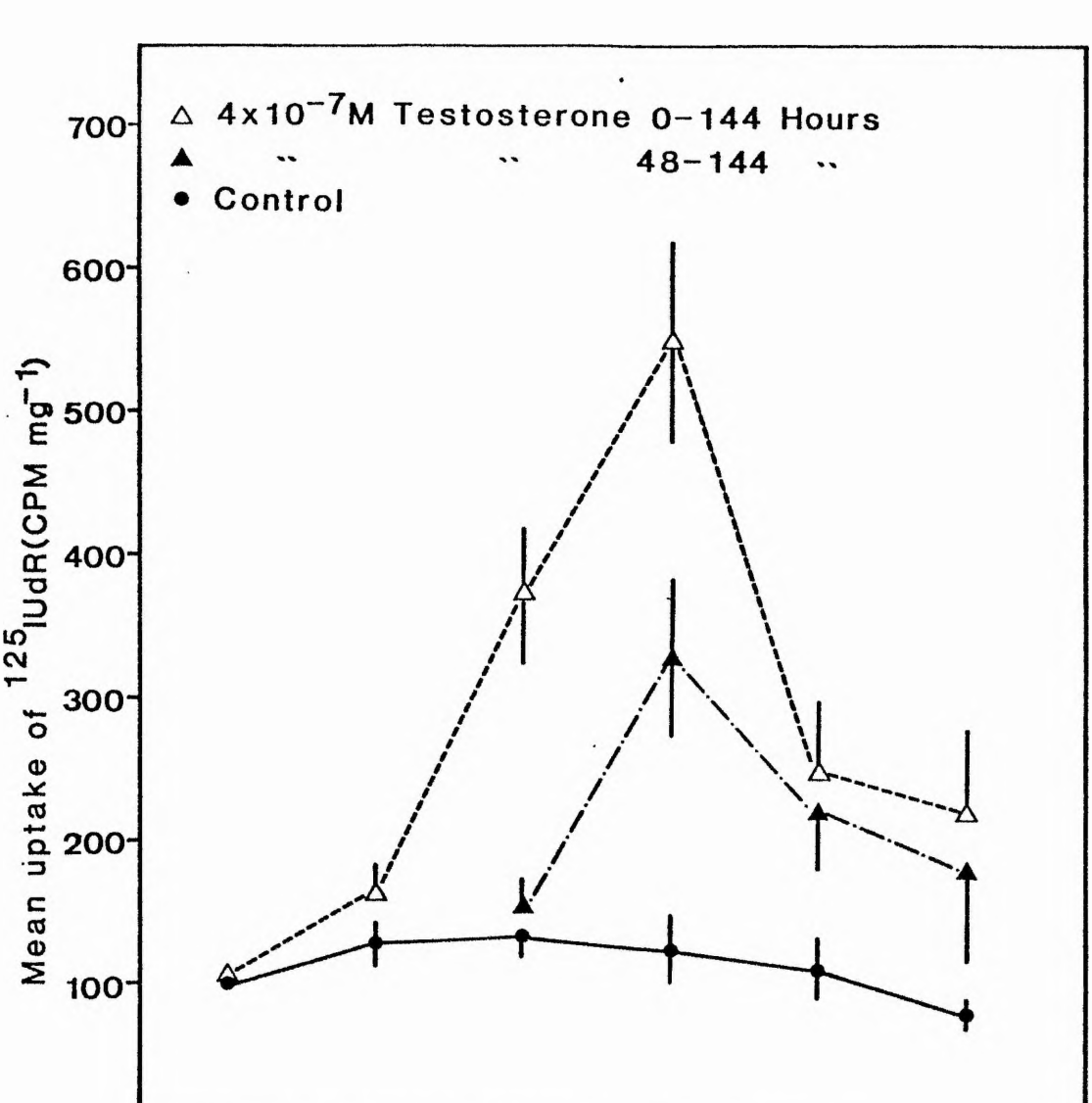

△ 4x10⁻⁷M Testosterone 0–144 Hours
▲ " " 48–144 "
● Control
Mean uptake of 125IUdR(CPM mg⁻¹)
700
600
500
400
300
200
100
1
2
3
4
5
6
DAYS IN CULTURE

Fig. 3.9 Effects of insulin ($3\mu g\ ml^{-1}$) and/or 5% foetal calf serum (FCS) on the incorporation of ^{125}I-UdR by explants of young adult rat ventral prostate following four days of organ culture in the absence (Control) and presence of testosterone (4×10^{-7} and $4 \times 10^{-5}M$).

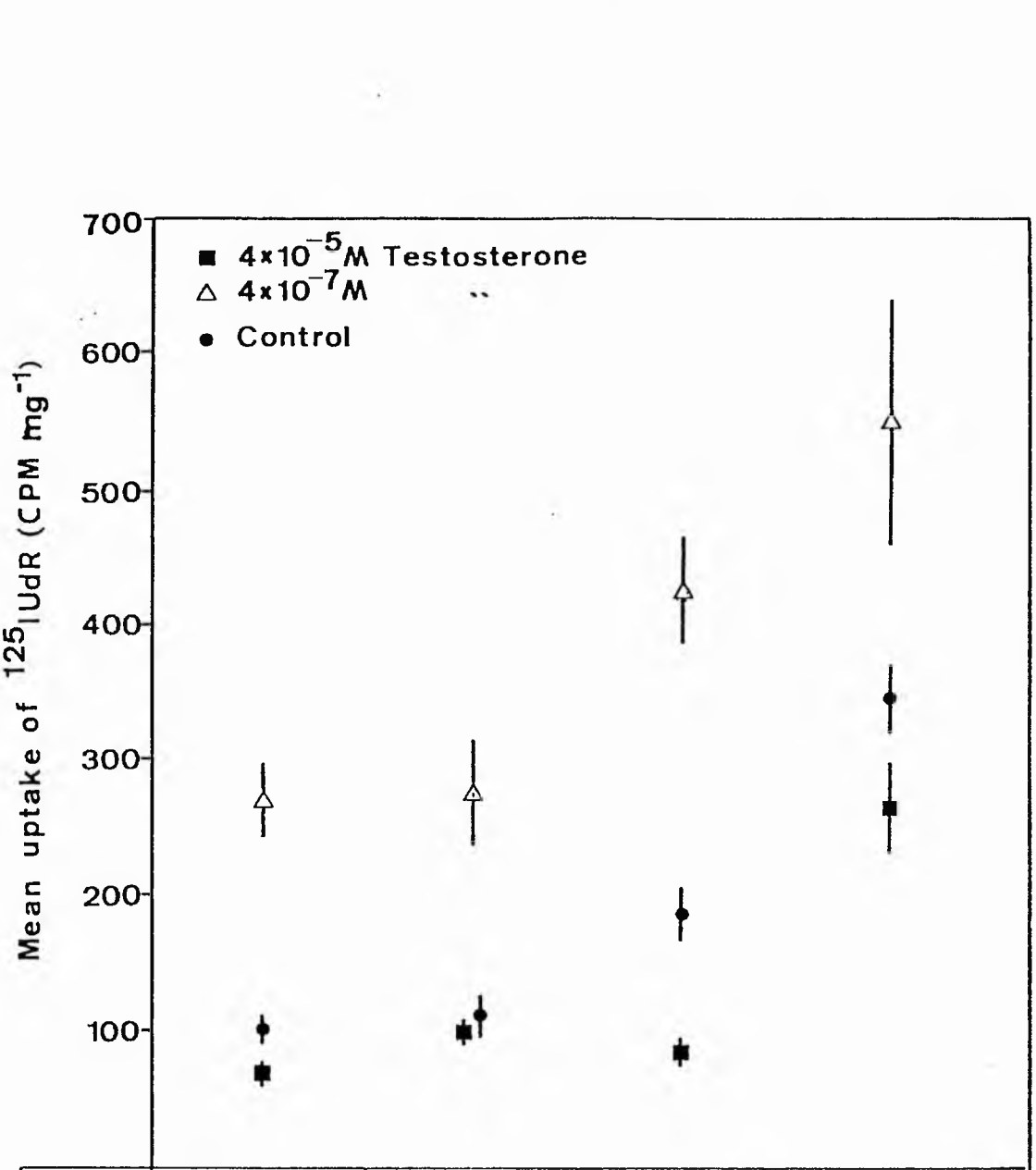

700
600
500
400
300
200
100
Mean uptake of ^{125}IUdR (CPM mg^{-1})
■ 4×10^{-5}M Testosterone
△ 4×10^{-7}M
● Control
INSULIN — — 3 µg ml^{-1} 3 µg ml^{-1}
SERUM — 5% FCS — 5% FCS

Fig. 3.10 Incorporation of ^{125}I-UdR by explants of young adult rat ventral prostate following four days of organ culture on Millipore filter paper (PAPER), directly on the grid (GRID) or submerged in chemically-defined medium (BOTTOM), either in the absence (Control) or presence of testosterone (4×10^{-7}M).

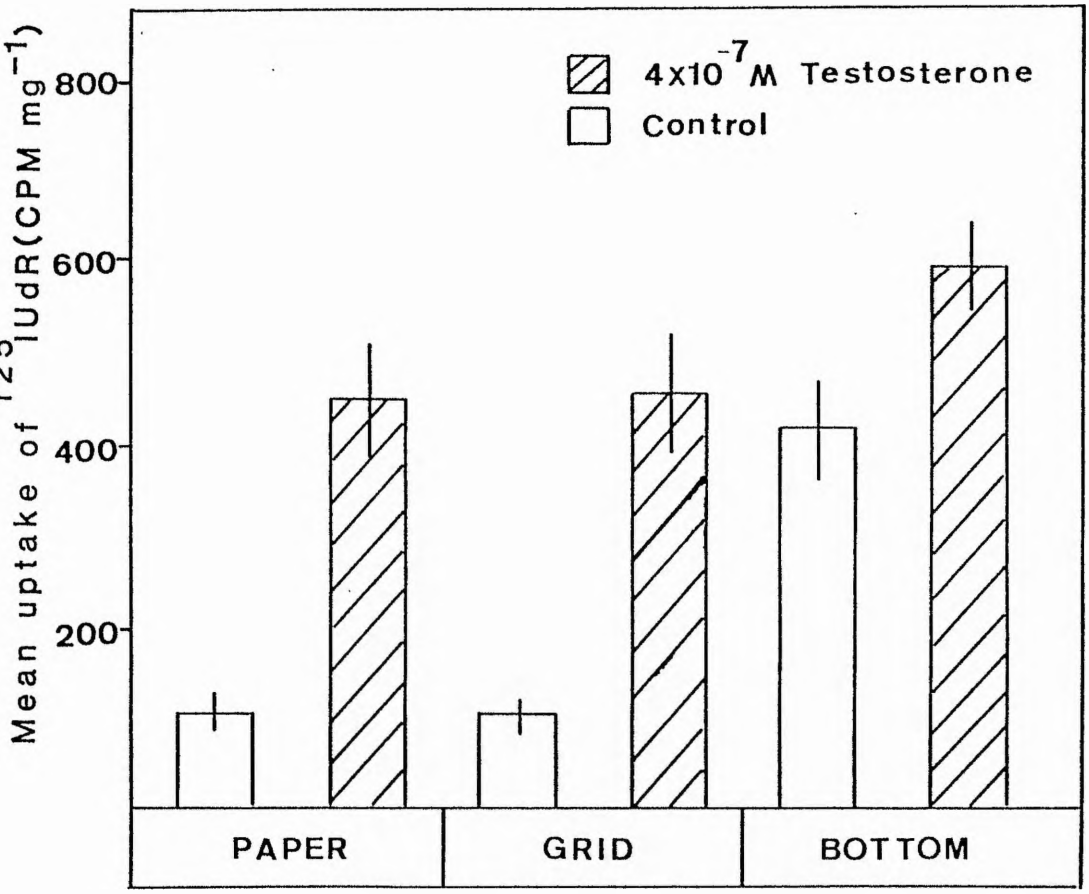

Mean uptake of ^{125}IUdR(CPM mg^{-1})
800
600
400
200
4x10^{-7}M Testosterone
Control
PAPER
GRID
BOTTOM

Fig. 3.11 Incorporation of ^{125}I-UdR by explants of young adult rat ventral prostate following four days of organ culture in chemically-defined medium in the presence of testosterone (T) and 5α-dihydro-testosterone (DHT) at concentrations of 4×10^{-9}, 4×10^{-7} and 4×10^{-5}M. Androgen-free control cultures are represented by ■, medium only, and □, medium supplemented with the alcohol diluent.

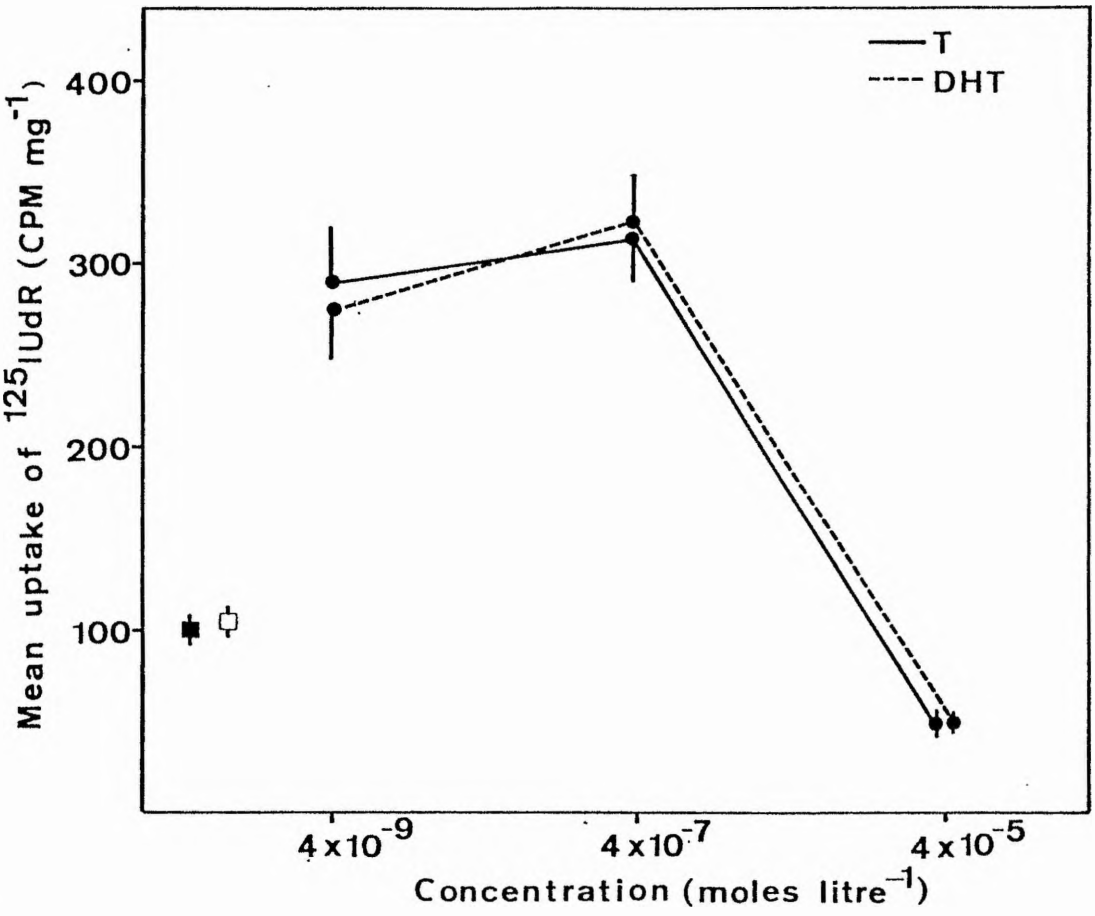

T
DHT
400
300
200
100
Mean uptake of ^{125}IUdR (CPM mg^{-1})
4x10^{-9}
4x10^{-7}
4x10^{-5}
Concentration (moles litre^{-1})

Fig. 3.12 Incorporation of ^{125}I-UdR by explants of young adult rat ventral prostate following four days of organ culture in chemically-defined medium in the presence of 5α-dihydrotestosterone (DHT) and 5β-dihydrotestosterone (5β-DHT) at concentrations of 4×10^{-9}, 4×10^{-7} and 4×10^{-5}M. Androgen-free control cultures are represented by ■, medium only, and □, medium supplemented with the alcohol diluent.

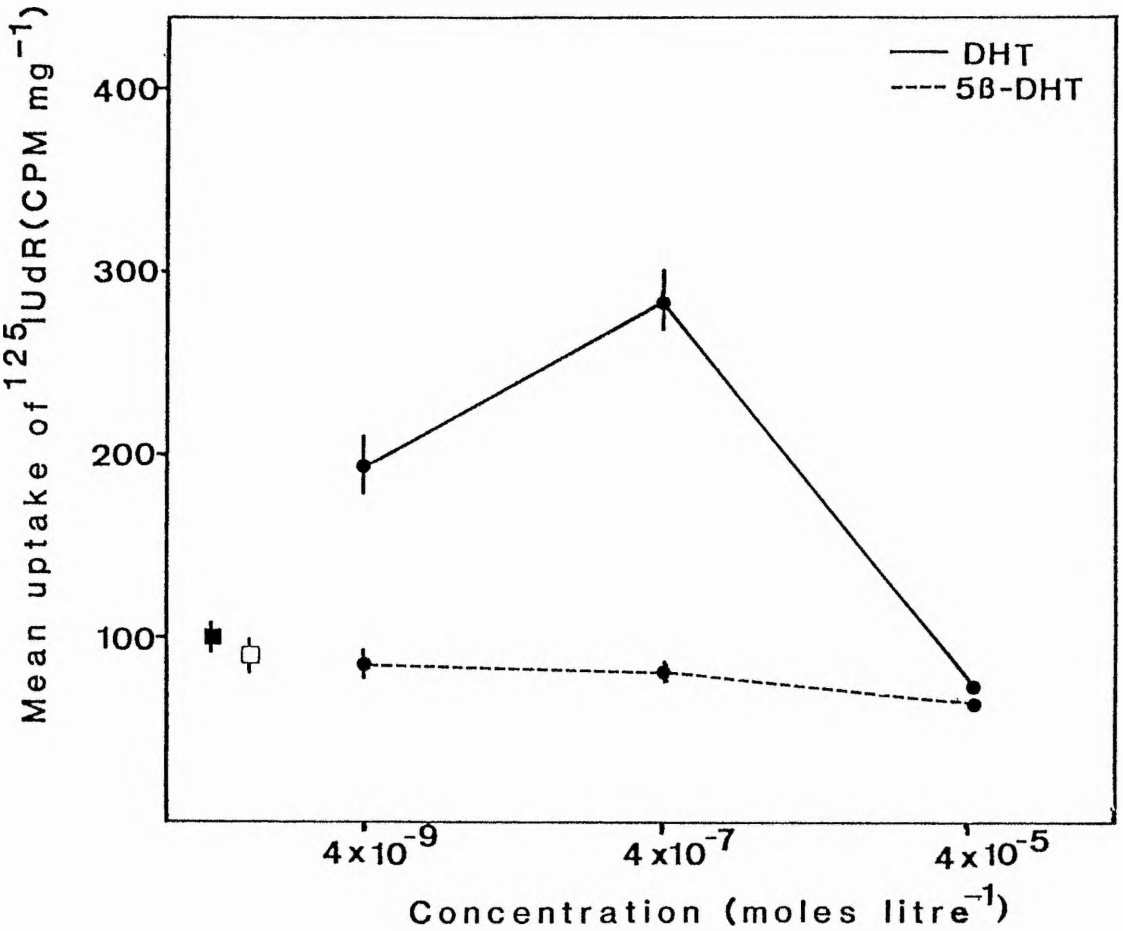

DHT
5β-DHT
400
300
200
100
Mean uptake of ^{125}IUdR(CPM mg^{-1})
4x10^{-9}
4x10^{-7}
4x10^{-5}
Concentration (moles litre^{-1})

Fig. 3.13 Incorporation of ^{125}I-UdR by explants of young adult rat ventral prostate following four days of organ culture in chemically-defined medium in the presence of testosterone (T), androstanedione (A-3,17-dione), androstenedione (Δ^4-A-3,17-dione) and androstane-3β, 17β-diol (A-3β,17β-diol) at concentrations of 4×10^{-9}, 4×10^{-7} and 4×10^{-5}M. Androgen-free control cultures are represented by ■, medium only, and □, medium supplemented with the alcohol diluent.

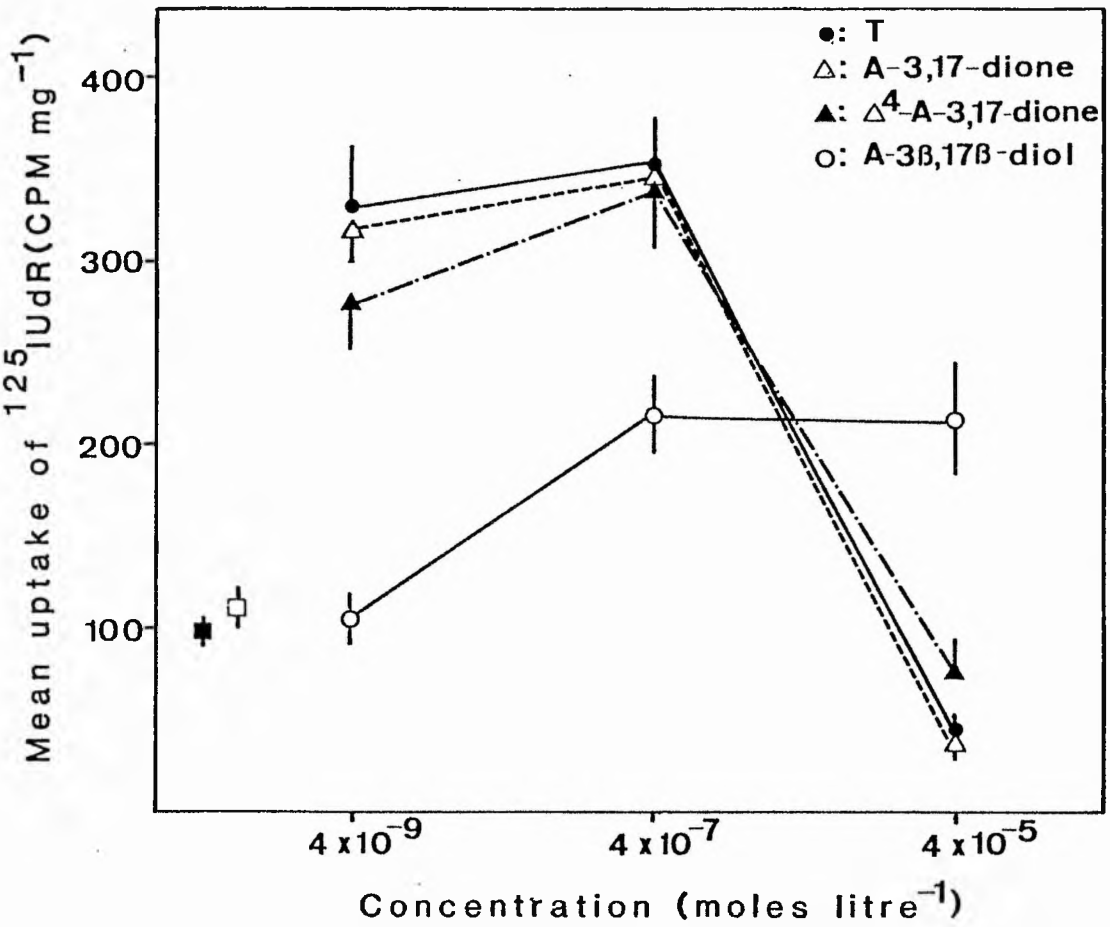

●: T
△: A-3,17-dione
▲: △4-A-3,17-dione
○: A-3β,17β-diol
Mean uptake of 125IUdR(CPM mg−1)
400
300
200
100
4 x10−9
4 x10−7
4 x10−5
Concentration (moles litre−1)

Fig. 3.14 Incorporation of ^{125}I-UdR by explants of ventral prostate from retired breeding rats following four days of organ culture in chemically-defined medium in the absence (■, medium only; □, medium and alcohol diluent) and presence of testosterone (4×10^{-12}, 4×10^{-10}, 4×10^{-9}, 4×10^{-8}, 4×10^{-7}, 4×10^{-6} and 4×10^{-5}M).

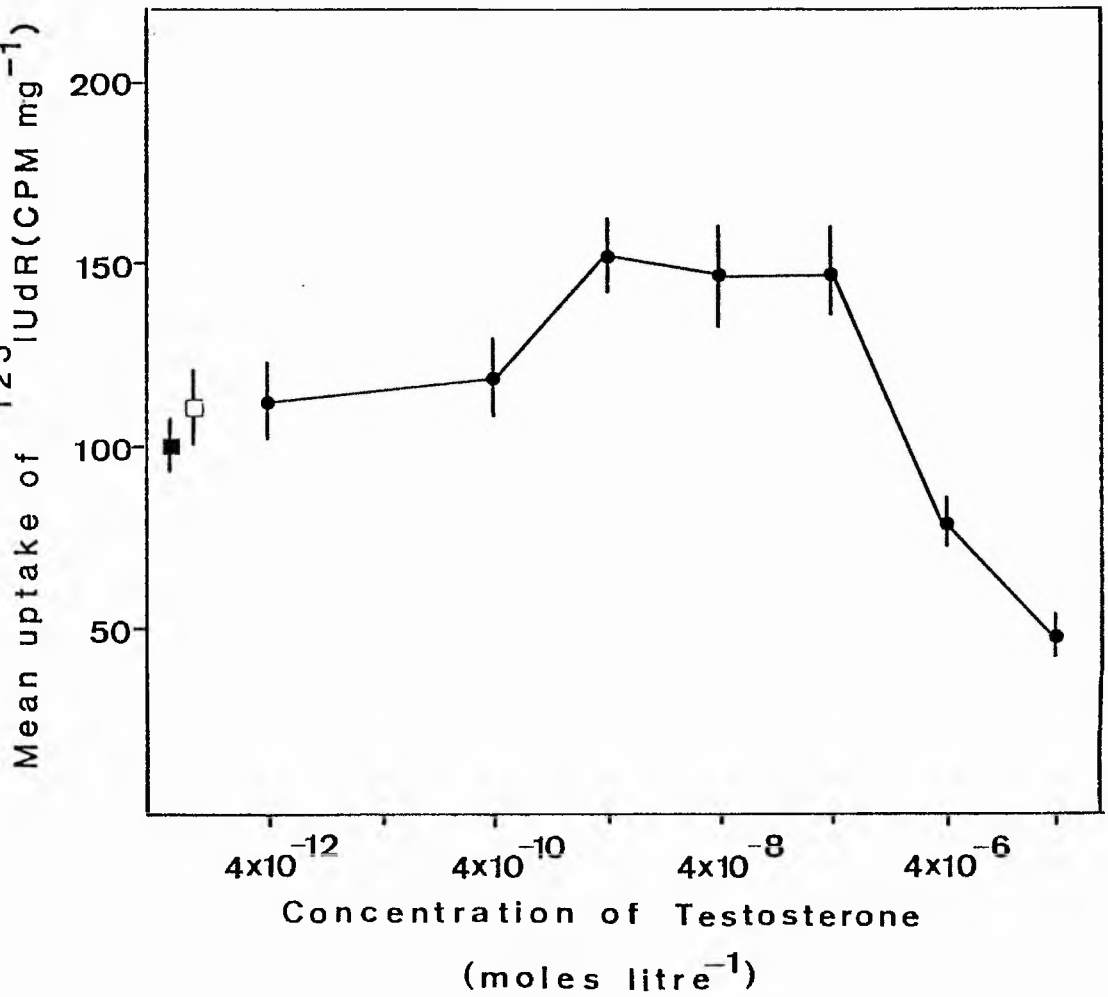

Mean uptake of ^{125}IUdR(CPM mg^{-1})
200
150
100
50
4x10^{-12}
4x10^{-10}
4x10^{-8}
4x10^{-6}
Concentration of Testosterone
(moles litre^{-1})

Fig. 3.15 Incorporation of ^{125}I-UdR by explants of ventral prostate from retired breeding rats cultured in chemically-defined medium for up to six days in the absence (Control) and presence of 4×10^{-7}M testosterone.

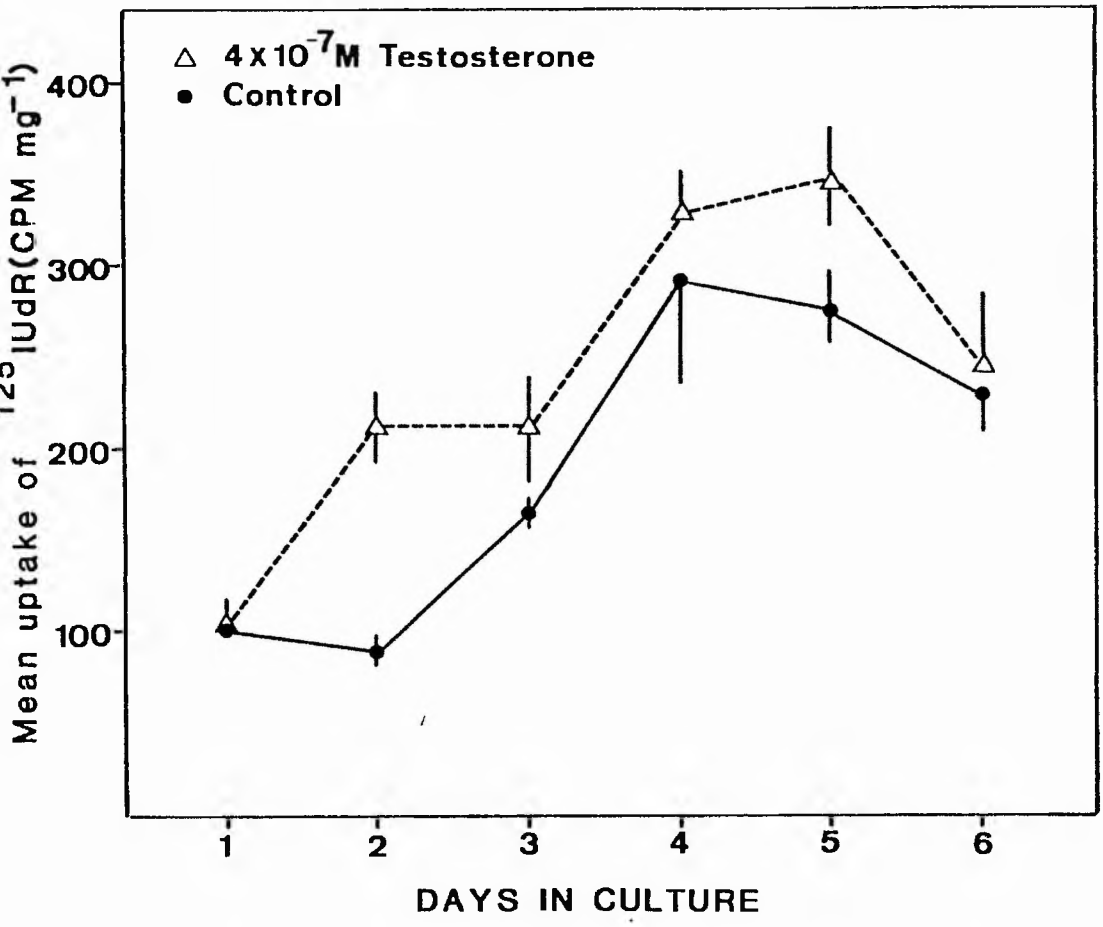

△ 4x10⁻⁷M Testosterone
● Control
Mean uptake of ¹²⁵IUdR(CPM mg⁻¹)
400
300
200
100
1
2
3
4
5
6
DAYS IN CULTURE

Plate 3.1 Alveoli in a fresh-fixed biopsy specimen of young adult rat ventral prostate showing actively secreting columnar epithelium. Haematoxylin & Eosin. X400.

Plate 3.2 Alveoli in a similar explant of young adult rat ventral prostate cultured for four days in non-supplemented, chemically-defined medium, showing low atrophic epithelium and absence of active secretion. Haematoxylin & Eosin. X400.

Plate 3.3 Alveoli in a similar explant cultured for four days in chemically-defined medium supplemented with 4×10^{-12}M testosterone showing reduced epithelial height and lack of fresh secretion, typical of androgen-free control cultures. Haematoxylin & Eosin. X400.

Plate 3.4 Alveoli in a similar explant cultured for four days in chemically-defined medium supplemented with 4×10^{-9}M testosterone showing well-maintained, actively secreting columnar epithelium, typical of the control before explantation (Plate 3.1). Haematoxylin & Eosin. X400.

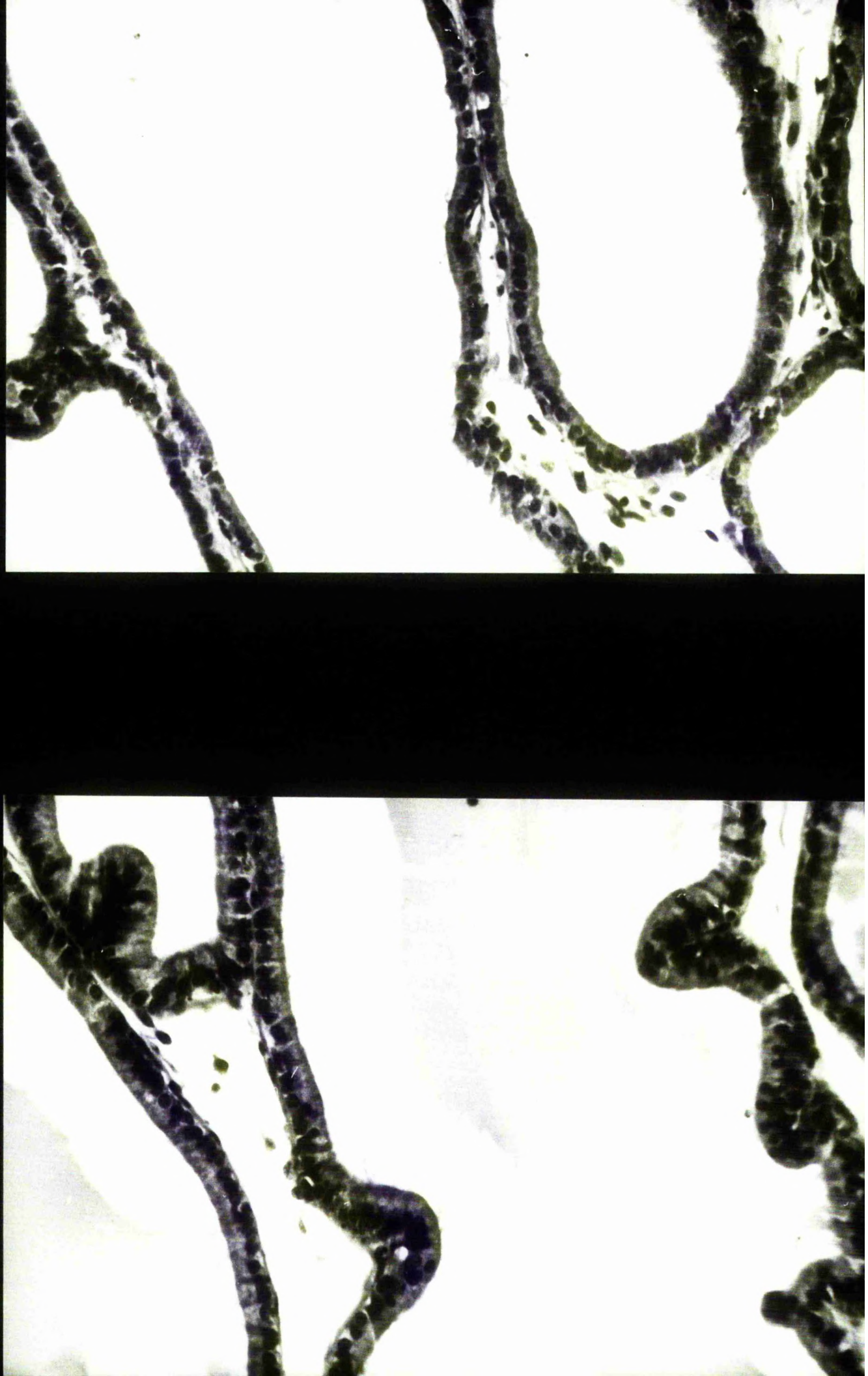
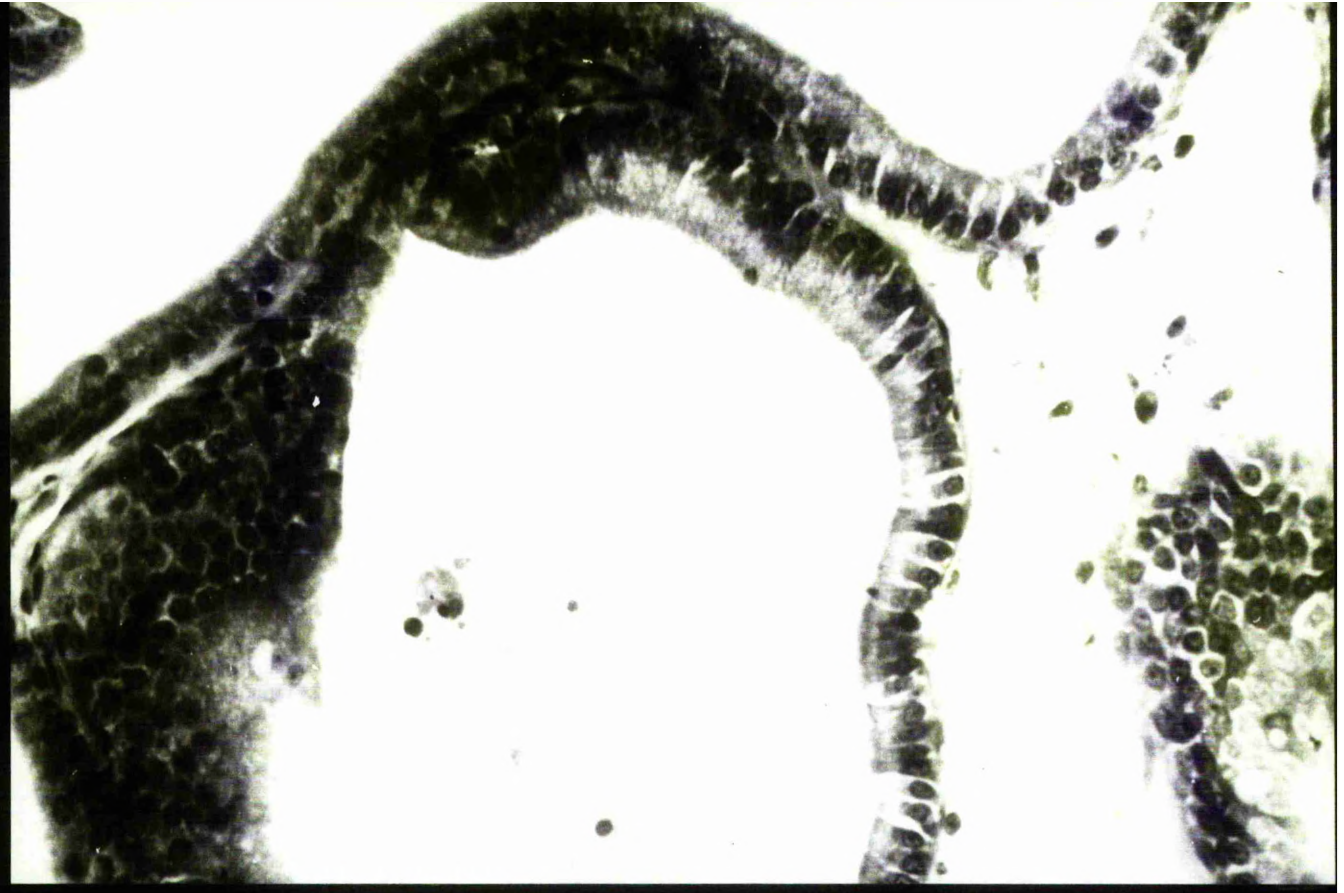


Plate 3.5 Alveoli in a similar explant cultured for four days in chemically-defined medium supplemented with 4×10^{-7}M testosterone showing actively secreting columnar epithelium and regions of epithelial hyperplasia. Haematoxylin & Eosin. X400.

Plate 3.6 Alveoli in a similar explant cultured for four days in chemically-defined medium supplemented with 4×10^{-5}M testosterone showing extensive necrosis of the alveolar epithelium and absence of fibromuscular stroma. Haematoxylin & Eosin. X400.



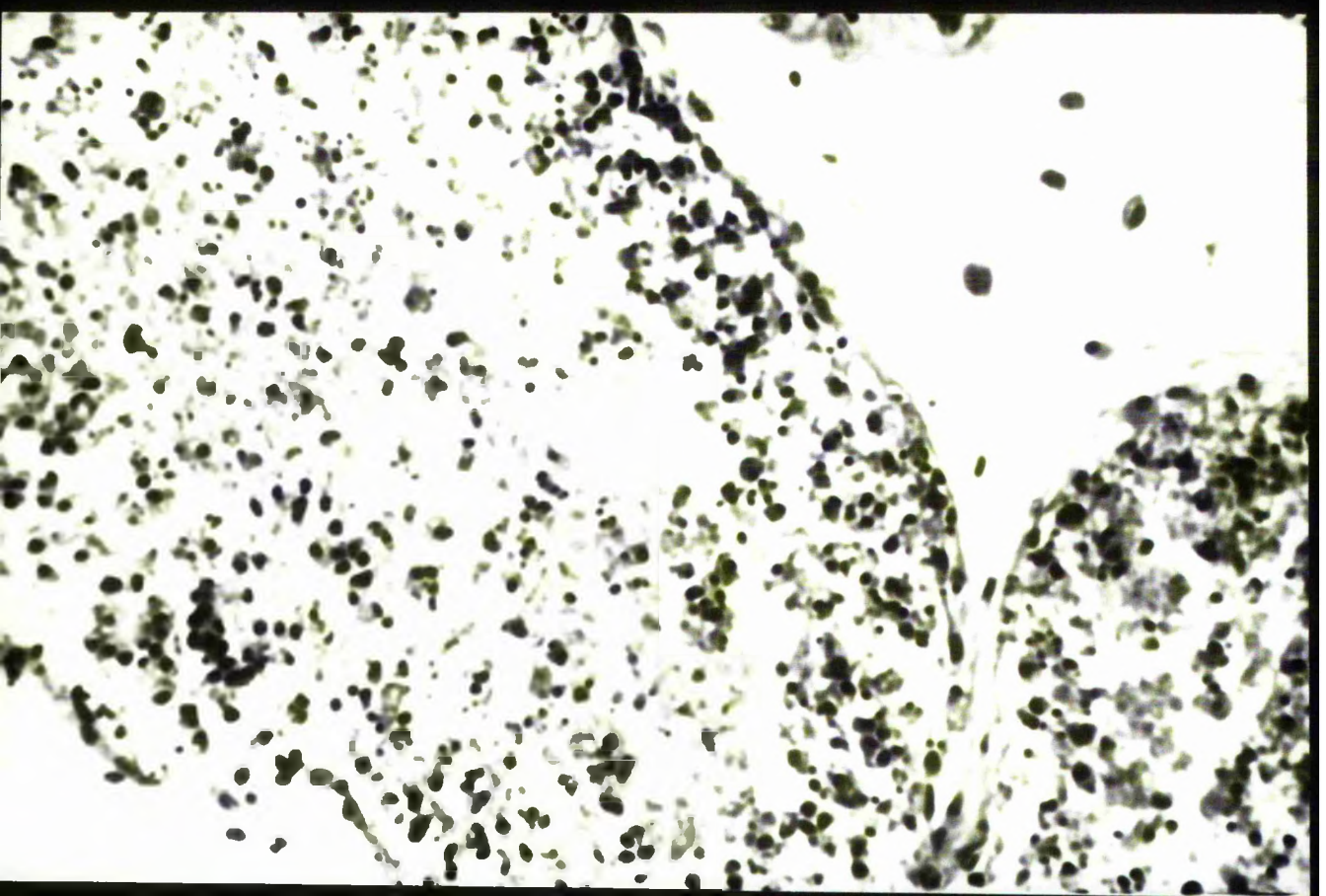


Plate 3.7 Autoradiograph of an explant of young adult rat ventral prostate cultured for four days in non-supplemented, chemically-defined medium showing the predominance of ^{3}H-TdR labelling over outgrowths of peripherally-located alveolar epithelium. Mayer's haemalum & Eosin. X400.

Plate 3.8 Autoradiograph of a similar explant cultured for four days in chemically-defined medium supplemented with 4×10^{-7}M testosterone showing the distribution of ^{3}H-TdR labelling over basal regions of the alveolar epithelium. Mayer's haemalum & Eosin. X400.

Plate 3.9 Alveoli in an explant of young adult rat ventral prostate cultured for four days in chemically-defined medium supplemented with 4×10^{-7}M 5α-dihydrotestosterone, showing well-maintained columnar epithelium and regions of hyperplastic growth. Haematoxylin & Eosin. X400.

Plate 3.10 Alveoli in a similar explant cultured for four days in chemically-defined medium supplemented with 4×10^{-5}M androstane-3β,17β-diol, showing well-maintained, actively secreting columnar epithelium. Haematoxylin & Eosin. X400.

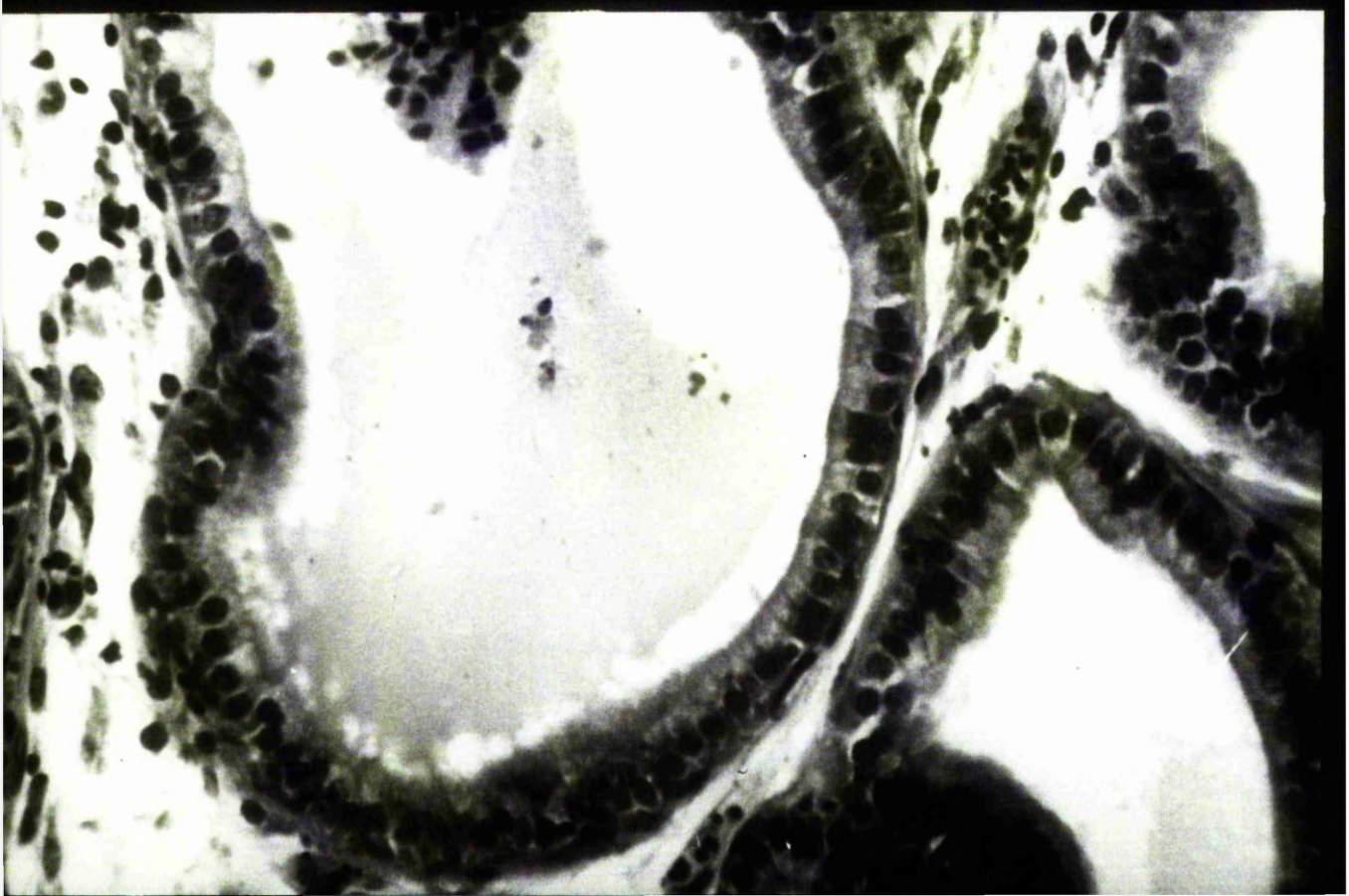
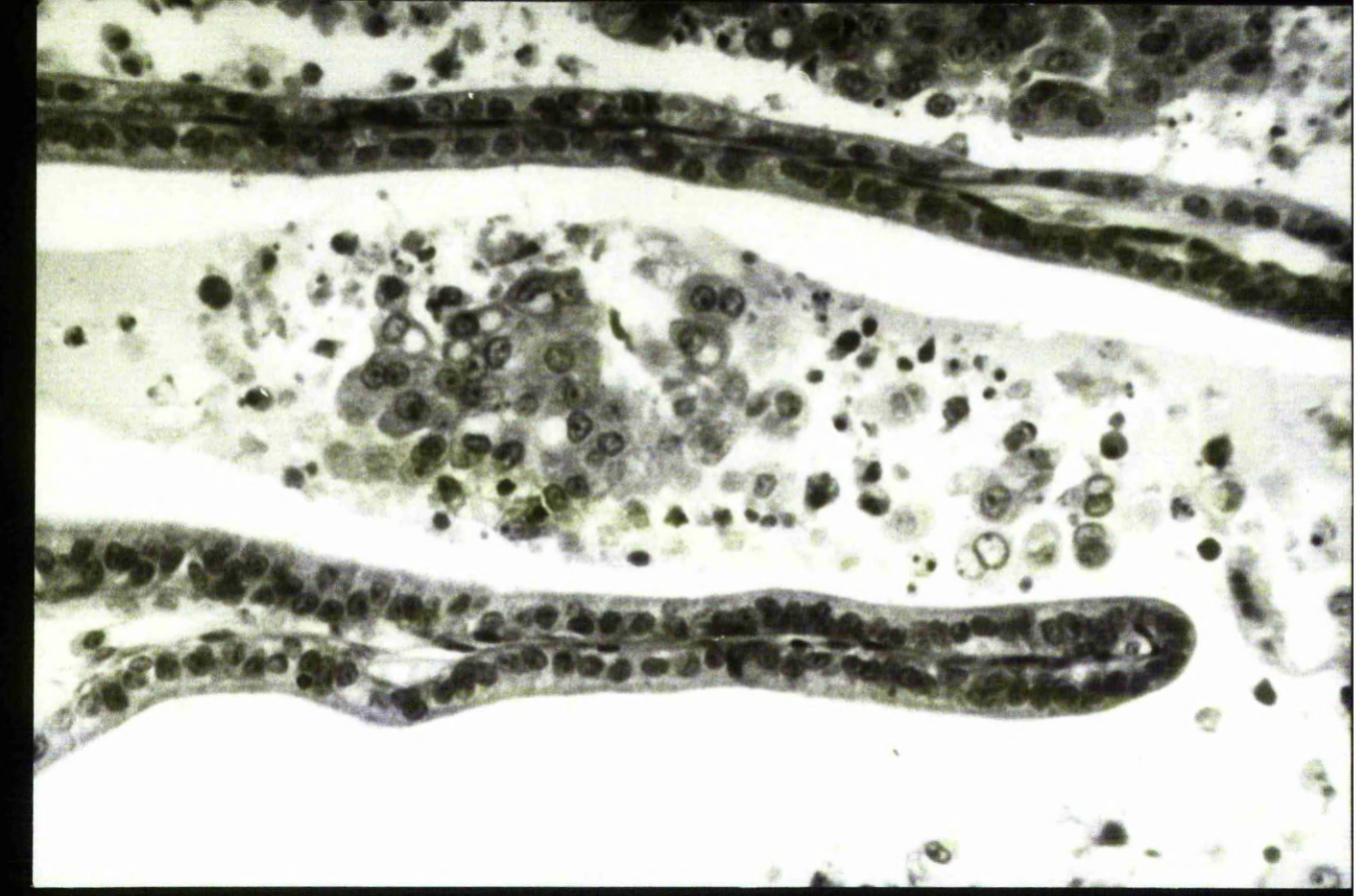


Plate 3.11 Alveoli in a similar explant cultured for four days in chemically-defined medium supplemented with 4×10^{-7}M 5β-dihydrotestosterone, showing low atrophic epithelium typical of androgen-deprived cultures. Haematoxylin & Eosin. X400.

Plate 3.12 Alveoli in a fresh-fixed biopsy specimen of ventral prostate from a retired breeding rat (> 12 months old), showing the heterogeneity of the alveolar epithelium and presence of concretions in the lumina. Haematoxylin & Eosin. X200.



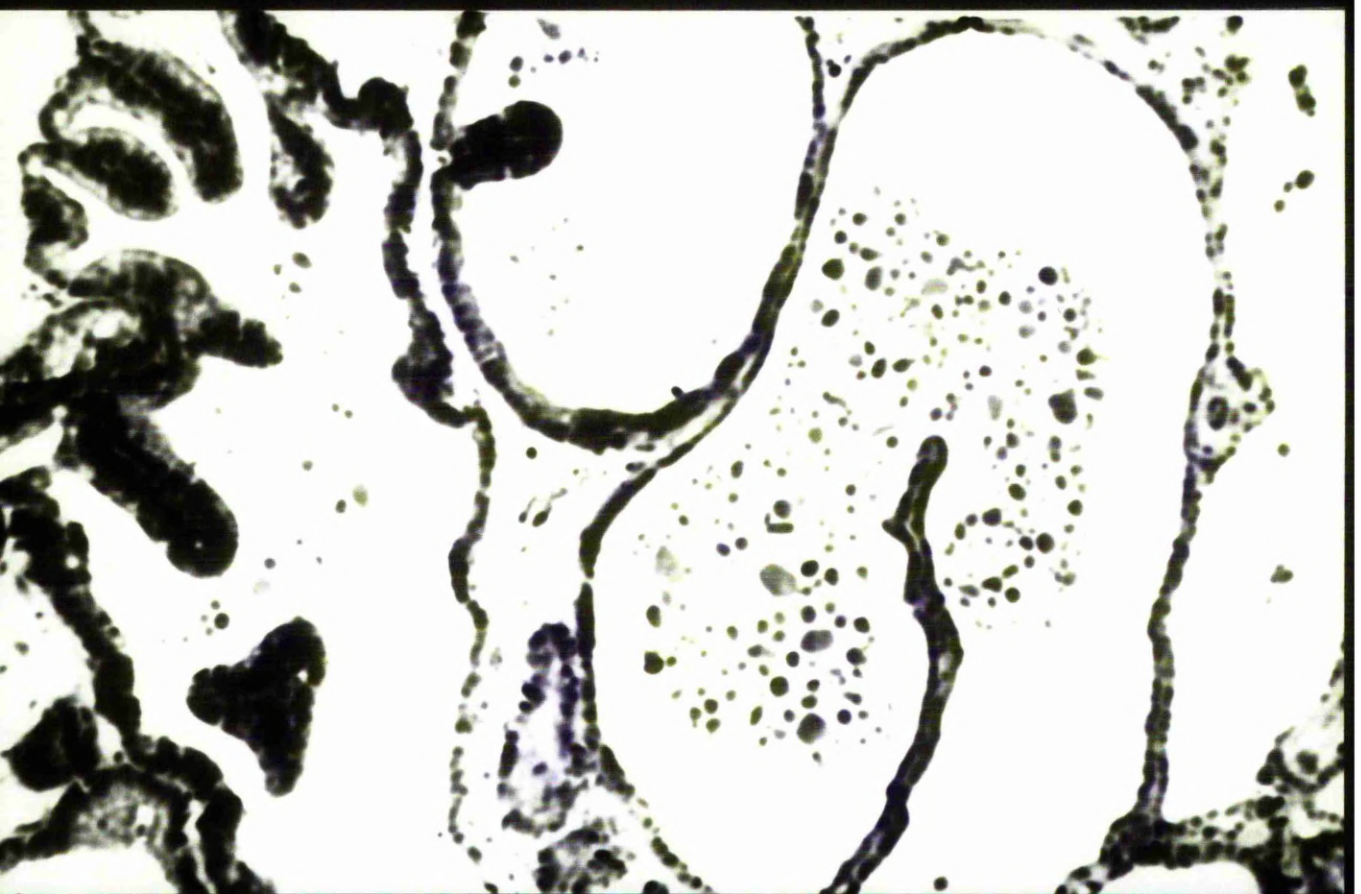
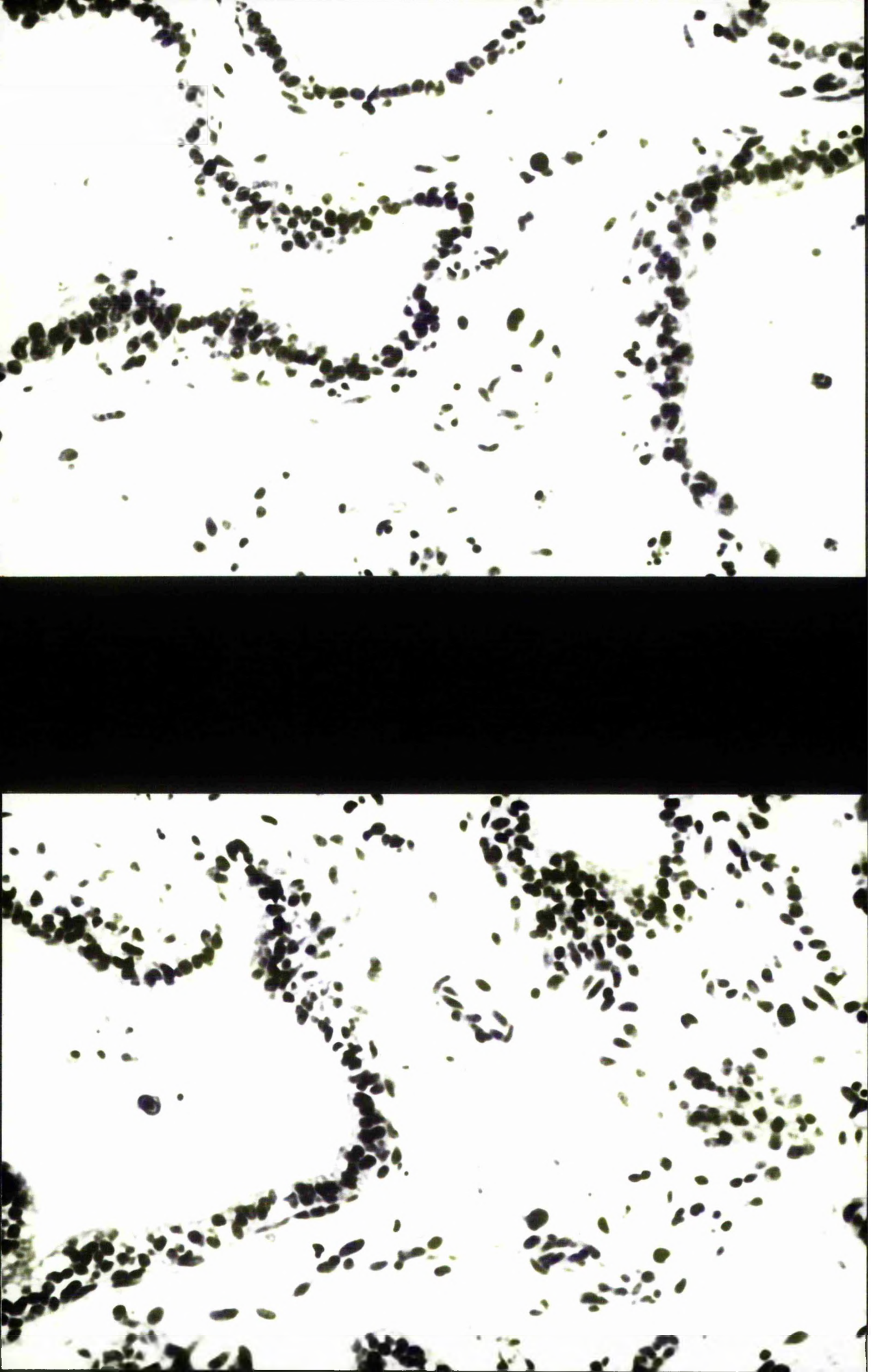


Plate 3.13 Alveoli in a similar explant of aged rat ventral prostate cultured for four days in non-supplemented, chemically-defined medium, showing irregularity of the alveolar epithelium, some regions of epithelial hyperplasia and degeneration of the fibromuscular stroma. Haematoxylin & Eosin. X400.

Plate 3.14 Alveoli in a similar explant cultured for four days in chemically-defined medium supplemented with 4×10^{-7}M testosterone, showing the same irregular appearance of the alveolar epithelium and degeneration of the stroma as seen in non-supplemented, control cultures of aged rat ventral prostate. Haematoxylin & Eosin. X400.



3.2 EFFECTS OF CHEMOTHERAPEUTIC AGENTS ON THE PROLIFERATIVE RESPONSE OF RAT VENTRAL PROSTATE TO TESTOSTERONE STIMULATION IN ORGAN CULTURE

Quantitative organ culture was used to evaluate the effects of antiandrogens, oestrogens and hormone-cytotoxic agents on testosterone-induced DNA synthesis in young adult rat ventral prostate. Explants were labelled with ^{125}I-UdR for the final 24h of a four day culture period and ^{125}I-UdR uptake has been expressed as:

$$\text{STIMULATION INDEX} = \frac{^{125}\text{I-UdR uptake of treatment group (CPM mg}^{-1})}{^{125}\text{I-UdR uptake of testosterone control (CPM mg}^{-1})}$$

such that the stimulation index of the testosterone control equals 1.0.

Results are presented as graphs and photomicrographs which are located at the end of section 3.2.

3.2.1 Effects of Cyproterone Acetate, Diethylstilboestrol, Oestradiol-17β and Estramustine Compounds on the Proliferative Response to 4 x 10^{-7}M Testosterone

^{125}I-UdR Results

The effect of cyproterone acetate at concentrations of 4 x 10^{-9}, 4 x 10^{-7} and 4 x 10^{-5}M on the proliferative response stimulated by 4 x 10^{-7}M testosterone is shown in Fig. 3.16. Testosterone and cyproterone acetate were added simultaneously on day 0 and replenished, following the medium change, on day 2. Treatment with 4 x 10^{-9} and 4 x 10^{-7}M cyproterone acetate did not significantly reduce ($p > 0.05$) the stimulatory effect of testosterone, whereas 4 x 10^{-5}M was significantly inhibitory ($p < 0.01$).

Using the same procedure, the effect of diethylstilboestrol on the testosterone-induced response was investigated (Fig. 3.17). Similarly, concentrations of 4×10^{-9} and 4×10^{-7}M diethylstilboestrol did not reduce the stimulatory response to testosterone, but at 4×10^{-5}M it was significantly inhibitory ($p < 0.01$).

The influence of oestradiol-17β, estramustine (LS 275), estramustine phosphate (LS 299) and estramustine phosphate sodium (LS 299Z) on the testosterone stimulated response were compared using the same procedure (Fig. 3.18). At 4×10^{-9} and 4×10^{-7}M oestradiol-17β significantly ($p < 0.05$) reduced the testosterone effect, and at 4×10^{-5}M was markedly inhibitory ($p < 0.01$). In contrast, the estramustine compounds exhibited a similar effect at all concentrations used and were consistently less effective than oestradiol-17β in reducing the testosterone stimulated response.

The effects of the estramustine compounds and oestradiol-17β at 4×10^{-5}M were compared both in the absence and presence of 4×10^{-7}M testosterone (Fig. 3.19). The estramustine compounds all had a similar effect in the presence of testosterone and were not significantly different ($p > 0.05$) from the testosterone control. However, oestradiol-17β had a significant ($p < 0.01$) inhibitory effect on the testosterone response. In the absence of testosterone, there was no significant difference ($p > 0.05$) between the estramustine compounds, oestradiol-17β and the non-supplemented control.

The influence of oestradiol-17β and the estramustine compounds (4×10^{-5}M) on the stimulatory effect of testosterone (4×10^{-7}M) was then investigated with respect

to the time at which the drugs and testosterone were added. In Fig. 3.20, testosterone was added only on day 0 and the estramustine compounds and oestradiol-17β were introduced on day 2 following renewal of the culture medium. The three estramustine compounds showed the same response and were not significantly different ($p > 0.05$) from the testosterone control, whereas oestradiol-17β significantly inhibited ($p < 0.01$) testosterone stimulation.

In Fig. 3.21, the estramustine compounds and oestradiol-17β were added only on day 0 and testosterone was introduced following the medium change on day 2. The estramustine compounds all exhibited the same response and were not significantly different ($p > 0.05$) from the testosterone control. However, oestradiol-17β had a significant ($p < 0.01$) inhibitory effect on the testosterone-induced response.

Histological Observations

The histological appearance of young adult rat ventral prostate cultured for four days in the presence of 4×10^{-7}M testosterone (Plate 3.15) resembled those cultures which had been treated with testosterone and either 4×10^{-9} or 4×10^{-7}M cyproterone acetate (Plate 3.16). In most alveoli the secretory epithelium was well-maintained and areas of epithelial cell proliferation were evident. In contrast, treatment with 4×10^{-5}M cyproterone acetate appeared to have a cytotoxic effect on both the stromal and epithelial components of the tissue (Plate 3.17). Most of the

alveolar epithelium was necrotic, the lumina were filled with cellular debris and often only "shadows" of former alveoli were observed. Stromal tissue was either scarce or absent.

Cultures which were treated with 4×10^{-7}M testosterone alone (Plate 3.18) or in combination with 4×10^{-9}, 4×10^{-7} or 4×10^{-5}M estramustine phosphate sodium, estramustine phosphate (Plate 3.19) or estramustine (Plate 3.20) were all histologically similar, showing good maintenance of the alveolar epithelium and secretory activity. In contrast, treatment with either 4×10^{-9} or 4×10^{-7}M oestradiol-17β caused slight regressive changes and 4×10^{-5}M oestradiol-17β caused extensive epithelial necrosis and stromal tissue was virtually absent (Plate 3.21). Similarly, treatment with the synthetic oestrogen, diethylstilboestrol, at 4×10^{-5}M was cytotoxic, but at lower concentrations it was inactive.

Cultures treated with the estramustine compounds (4×10^{-5}M) in the absence of testosterone underwent epithelial retrogression similar to that which occurred in non-supplemented control cultures. However, treatment with oestradiol-17β remained cytotoxic in the absence of testosterone.

Cultures treated with the estramustine compounds (4×10^{-5}M) either 48h before or after the introduction of 4×10^{-7}M testosterone were all well-maintained and histologically similar to the testosterone control, whereas treatment with 4×10^{-5}M oestradiol-17β was consistently cytotoxic.

3.2.2 Effects of Cyproterone Acetate, Oestradiol-17β and Estramustine Compounds on the Proliferative Response to 4×10^{-9}M Testosterone

^{125}I-UdR Results

The effect of cyproterone acetate at concentrations of 4×10^{-9}, 4×10^{-7} and 4×10^{-5}M on the response stimulated by 4×10^{-9}M testosterone is shown in Fig. 3.22. Testosterone and cyproterone acetate were added on day 0 and replenished on day 2 following the medium change. Cyproterone acetate at 4×10^{-9}M did not significantly reduce ($p > 0.05$) the stimulatory effect of testosterone, whereas higher concentrations (4×10^{-7} and 4×10^{-5}M) were increasingly inhibitory.

Using the same procedure, the influence of estramustine phosphate sodium, estramustine phosphate, estramustine and oestradiol-17β on the testosterone response were compared (Fig. 3.23). At 4×10^{-9} and 4×10^{-7}M, all the estramustine compounds elicited a similar response and only marginally reduced the stimulatory effect of testosterone, whereas at 4×10^{-5}M all the compounds were significantly inhibitory ($p < 0.01$). In contrast, oestradiol-17β significantly inhibited ($p < 0.01$) the testosterone stimulated response at all concentrations used.

The inhibitory effects of oestradiol-17β and the estramustine compounds (4×10^{-5}M) were investigated with regard to the time at which the drugs and testosterone were introduced. In Fig. 3.24, 4×10^{-9}M testosterone was added only on day 0 and addition of the drugs followed the medium change on day 2. All the estramustine compounds

reduced the testosterone response equally, but oestradiol-17β was the most effective inhibitor. By comparison with Fig. 3.23, delaying the onset of the drug addition appeared to reduce the magnitude of the inhibitory effects elicited by each compound.

In Fig. 3.25, the estramustine compounds and oestradiol-17β were added on day 0 only and testosterone was introduced on day 2. The estramustine compounds all had an equal, and marked, inhibitory effect on the testosterone stimulated response, but oestradiol-17β remained the most potent inhibitor. By comparison with Fig. 3.23, pre-treatment with the drugs did not appear to increase the magnitude of the inhibitory effects of any of the compounds.

Histological Observations

The histological appearance of rat ventral prostate cultured for four days in the presence of 4×10^{-9}M testosterone alone (Plate 3.22) or in combination with an equimolar concentration of cyproterone acetate were similar, showing good preservation of the tissue. However, treatment with 4×10^{-7}M cyproterone acetate (Plate 3.23) caused marked reductions in epithelial height and secretory activity in most alveoli. Treatment with 4×10^{-5}M cyproterone acetate had a cytotoxic effect on both the stroma and alveolar epithelium, similar to that shown in Plate 3.17.

In contrast, cultures treated with 4×10^{-9}M testosterone alone (Plate 3.24) or in combination with either 4×10^{-9} or 4×10^{-7}M estramustine phosphate sodium, estramustine phosphate or estramustine were all

histologically similar and well-maintained. However, in cultures treated with the estramustine compounds at a concentration of 4×10^{-5}M, most alveoli were dilated and lined with flattened epithelium. Epithelial folding was markedly reduced and secretory activity was diminished or absent (Plate 3.25). Unlike the estramustine compounds, however, treatment with either 4×10^{-9} or 4×10^{-7}M oestradiol-17β, in the presence of 4×10^{-9}M testosterone, caused severe epithelial atrophy and necrosis (Plate 3.26), and 4×10^{-5}M oestradiol-17β was cytotoxic.

Pre-treatment with the estramustine compounds (4×10^{-5}M) for 48h before the addition of 4×10^{-9}M testosterone caused epithelial retrogression, similar to that shown in Plate 3.25, whereas extensive epithelial and stromal necrosis occurred in cultures treated with 4×10^{-5}M oestradiol-17β.

Cultures treated with the estramustine compounds (4×10^{-5}M) 48h after the introduction of testosterone (4×10^{-9}M) were histologically similar to the testosterone control, whereas treatment with 4×10^{-5}M oestradiol-17β remained cytotoxic.

3.2.3 The Influence of Serum and the Effects of Oestradiol-17β and the Estramustine Compounds on the Proliferative Response to 4×10^{-6}M Testosterone

^{125}I-UdR Results

The effects of estramustine phosphate, estramustine and oestradiol-17β on the proliferative response stimulated by 4×10^{-6}M testosterone were compared in the presence and absence of 5% foetal calf serum (Fig. 3.26). Testosterone

(4×10^{-6}M) and the drugs (4×10^{-5}M) were added on day 0 and neither the medium nor the supplements were renewed during the four day culture period (Høisaeter, 1975b). In the serum-free system, there was no significant difference ($p > 0.05$) between the response of cultures treated with the estramustine compounds and the testosterone control, whereas treatment with oestradiol-17β was significantly inhibitory ($p < 0.01$). In contrast, all the drugs exhibited comparable responses and were significantly inhibitory ($p < 0.05$) in serum-supplemented medium.

Using the same procedure, the inhibitory effects of estramustine phosphate, estramustine and oestradiol-17β were compared in the absence and presence of 5, 10 and 20% foetal calf serum (Fig. 3.27). In the serum-free system, neither of the estramustine compounds had a significant inhibitory effect on the testosterone stimulated response, whereas oestradiol-17β was significantly inhibitory ($p < 0.01$). However, in the presence of 5, 10 or 20% foetal calf serum, the estramustine compounds and oestradiol-17β were all equally inhibitory. By comparison with the serum-free system, it appeared that the presence of serum enhanced the inhibitory effects of estramustine phosphate and estramustine, whereas it decreased the inhibitory action of oestradiol-17β.

Histological Observations

The histological appearance of cultures maintained in serum-free medium supplemented with 4×10^{-6}M testosterone alone (Plate 3.27) or in combination with 4×10^{-5}M estramustine phosphate (Plate 3.28) or estramustine were all well-preserved, whereas treatment

with 4 x 10^{-5}M oestradiol-17β was cytotoxic (Plate 3.29). In contrast, only cultures treated with 4 x 10^{-6}M testosterone were well-maintained in the presence of 5% foetal calf serum (Plate 3.30). Treatment with testosterone and 4 x 10^{-5}M estramustine phosphate (Plate 3.31) or estramustine in serum-supplemented medium caused epithelial retrogression which was frequently accompanied by necrosis. However, in serum-supplemented cultures treated with testosterone and 4 x 10^{-5}M oestradiol-17β most of the alveoli were dilated and lined with flattened, non-secreting epithelial cells (Plate 3.32). The effects of oestradiol-17β and the estramustine compounds on cultures maintained in medium containing higher concentrations of foetal calf serum (10 and 20%) were similar to that observed in the presence of 5% foetal calf serum.

Fig. 3.16 Effects of 4×10^{-9}, 4×10^{-7} and 4×10^{-5}M cyproterone acetate on the 4×10^{-7}M testosterone-stimulated response of rat ventral prostate on day 4 following organ culture in chemically-defined medium. Non-supplemented control cultures are represented by ■, medium only, and □, medium containing the alcohol diluent.

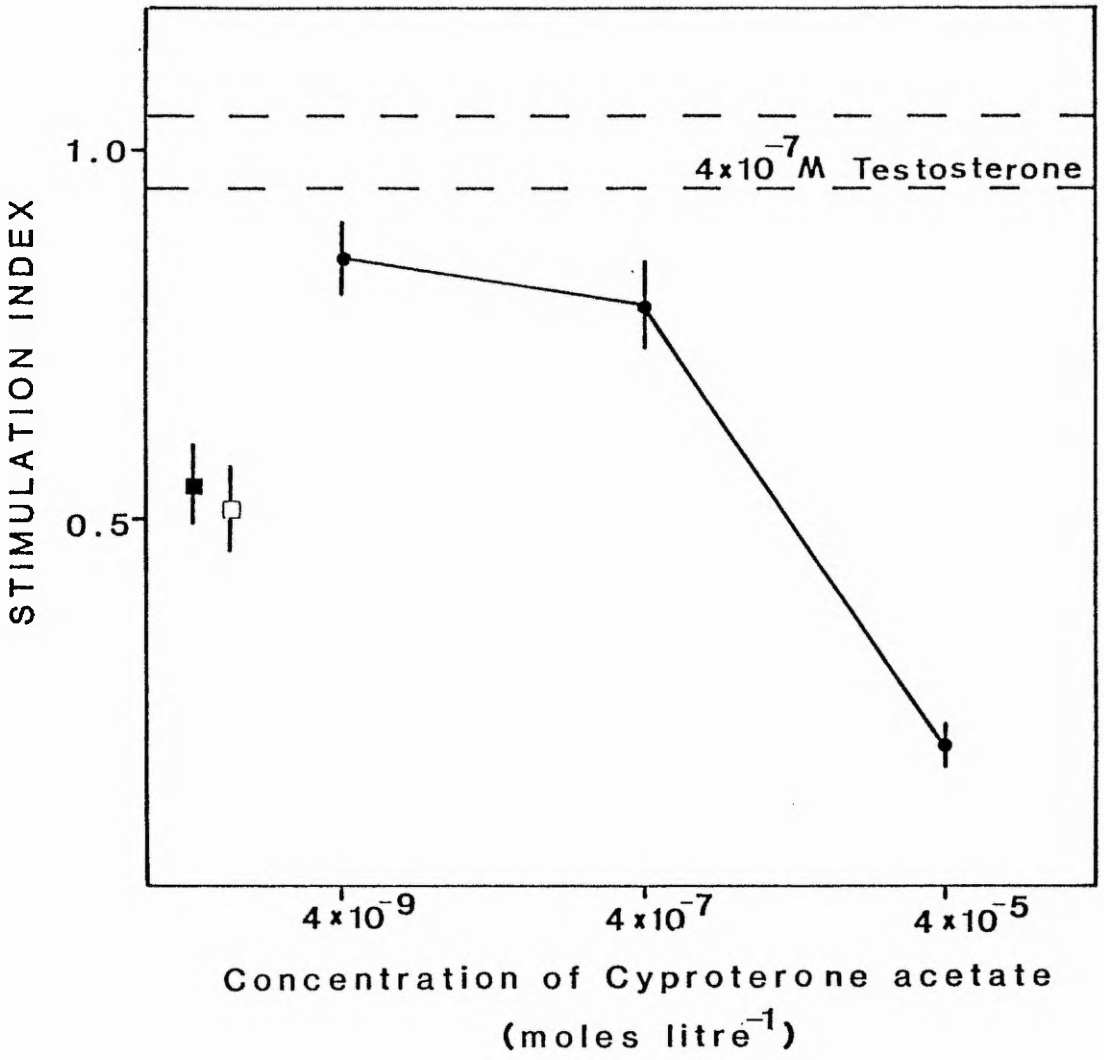

STIMULATION INDEX
1.0
0.5
4x10⁻⁷M Testosterone
4x10⁻⁹
4x10⁻⁷
4x10⁻⁵
Concentration of Cyproterone acetate
(moles litre⁻¹)

Fig. 3.17 Effects of 4×10^{-9}, 4×10^{-7} and 4×10^{-5}M diethylstilboestrol on the 4×10^{-7}M testosterone-stimulated response of rat ventral prostate on day 4 following organ culture in chemically-defined medium. Non-supplemented control cultures are represented by ■, medium only, and □, medium containing the alcohol diluent.

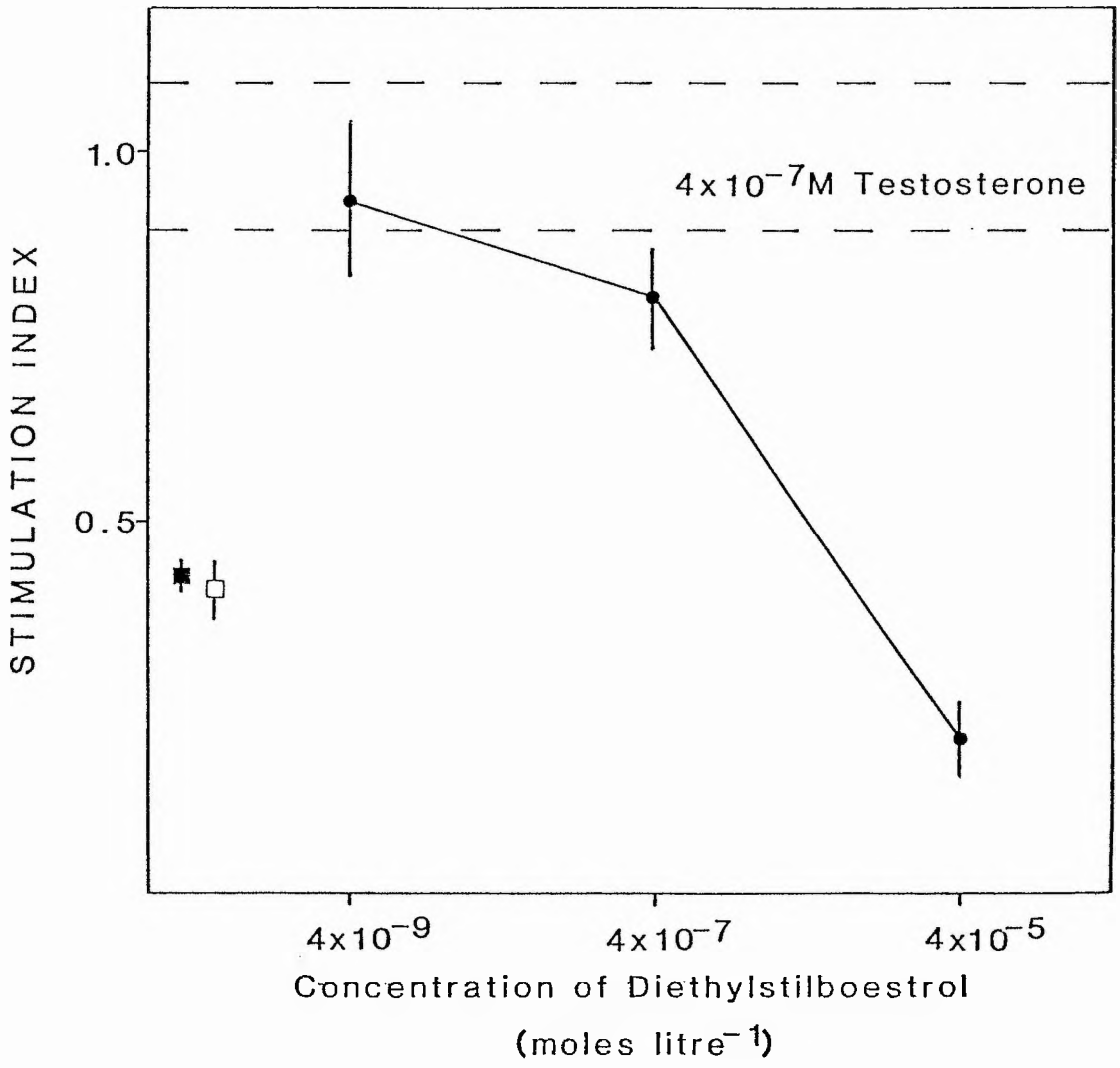

STIMULATION INDEX
1.0
0.5
4x10⁻⁷M Testosterone
4x10⁻⁹
4x10⁻⁷
4x10⁻⁵
Concentration of Diethylstilboestrol
(moles litre⁻¹)

Fig. 3.18 Effects of estramustine phosphate sodium (LS 299Z), estramustine phosphate (LS 299), estramustine (LS 275) and oestradiol-17β (E_2-17β) on the 4 x 10^{-7}M testosterone-stimulated response of rat ventral prostate on day 4 following organ culture in chemically-defined medium. Non-supplemented control cultures are represented by ■, medium only and □, medium containing the alcohol diluent.

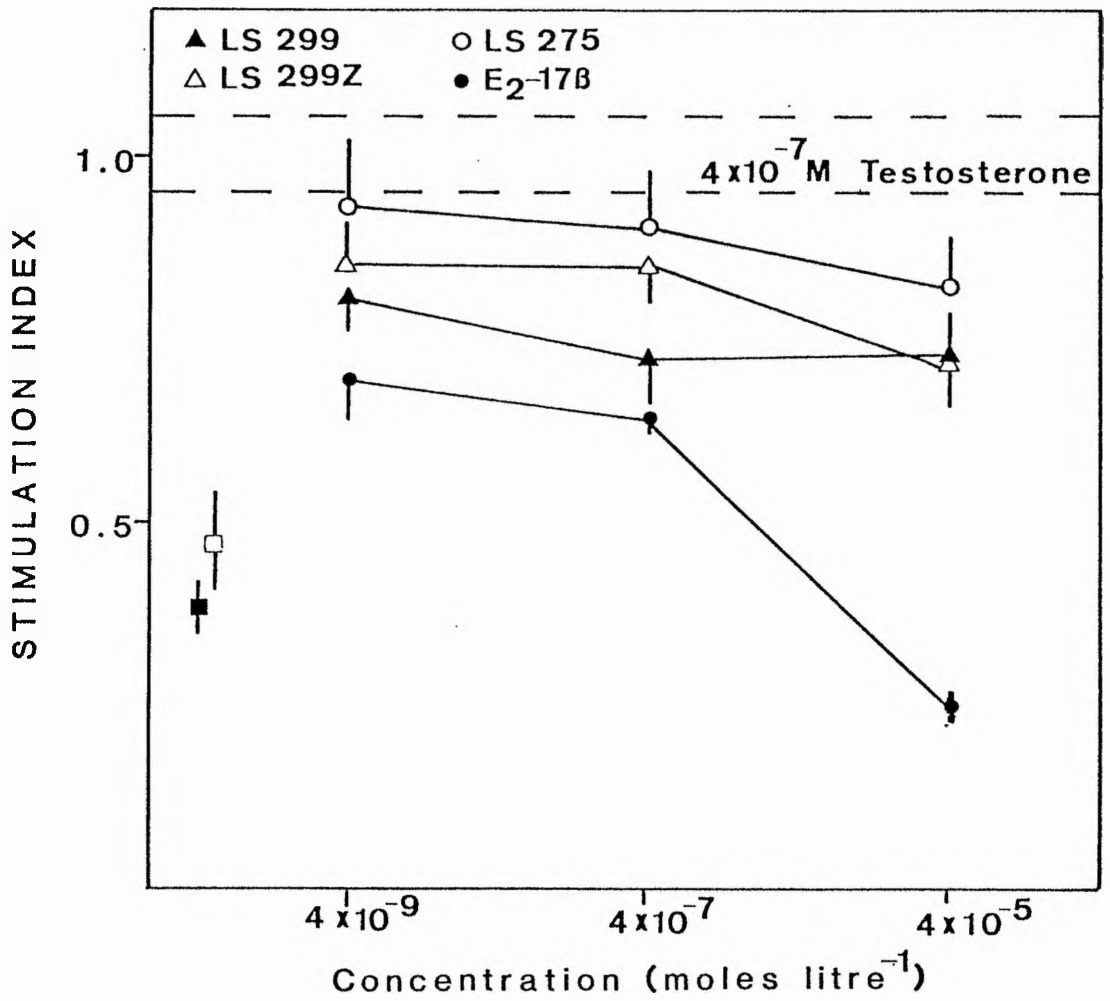

▲ LS 299
△ LS 299Z
○ LS 275
● E_2-17β
4×10^{-7} M Testosterone
1.0
0.5
STIMULATION INDEX
4×10^{-9}
4×10^{-7}
4×10^{-5}
Concentration (moles litre^{-1})

Fig. 3.19 Proliferative responses of rat ventral prostate on day 4 following organ culture in chemically-defined medium supplemented with 4×10^{-5}M estramustine phosphate sodium (LS 299Z), estramustine phosphate (LS 299), estramustine (LS 275) or oestradiol-17β (E_2-17β) in the absence (CONTROL) and presence of 4×10^{-7}M testosterone.

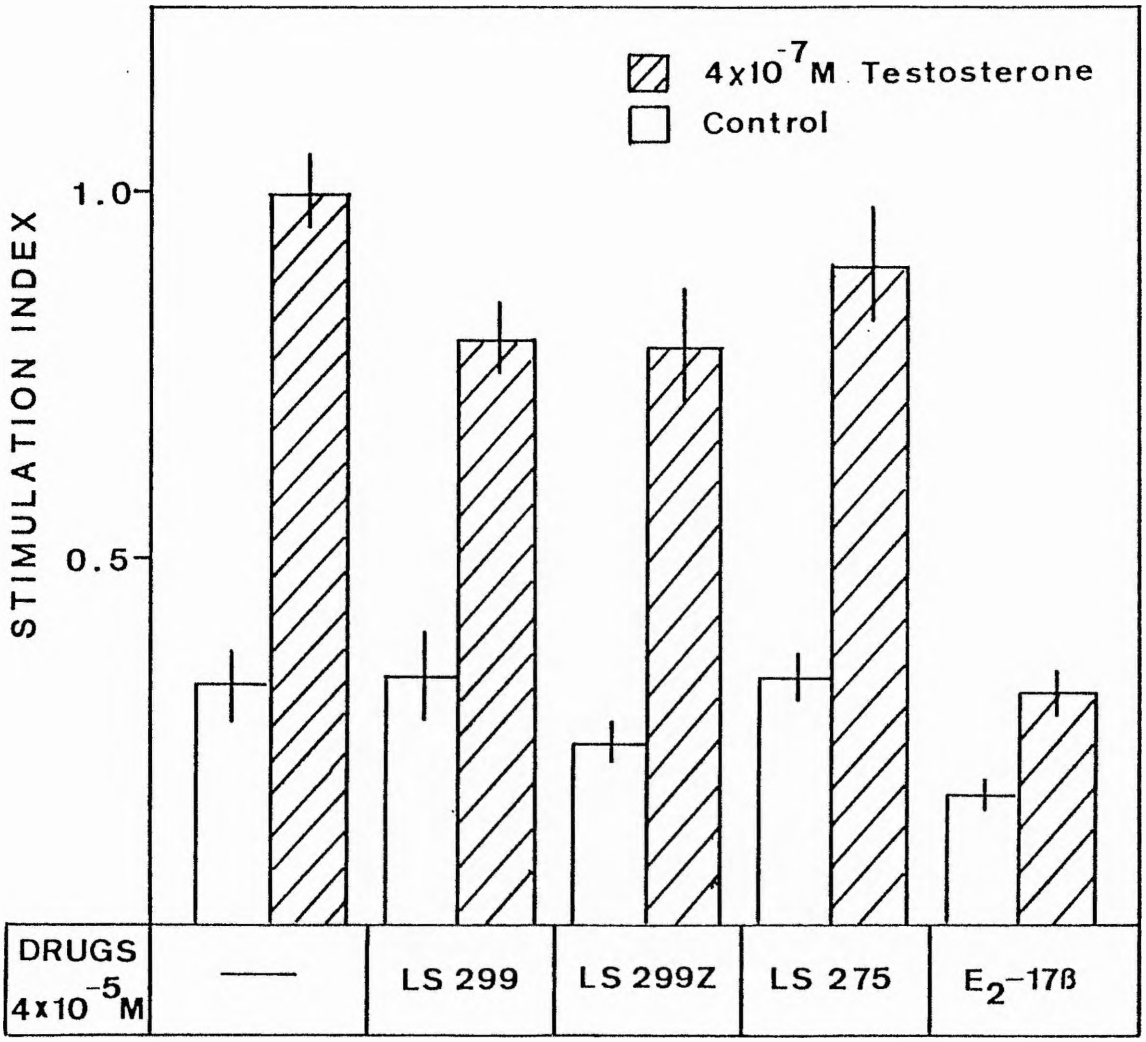

4x10⁻⁷M Testosterone
Control
STIMULATION INDEX
1.0
0.5
DRUGS
4x10⁻⁵M
—
LS 299
LS 299Z
LS 275
E2-17ß

Fig. 3.20 Proliferative responses of rat ventral prostate on day 4 of organ culture in chemically-defined medium following treatment with 4 x 10^{-7}M testosterone for the first 48h and 4 x 10^{-5}M estramustine phosphate (LS 299Z), estramustine phosphate (LS 299), estramustine (LS 275) or oestradiol-17β (E_2-17β) during the final 48h. 'Control' represents non-supplemented cultures.

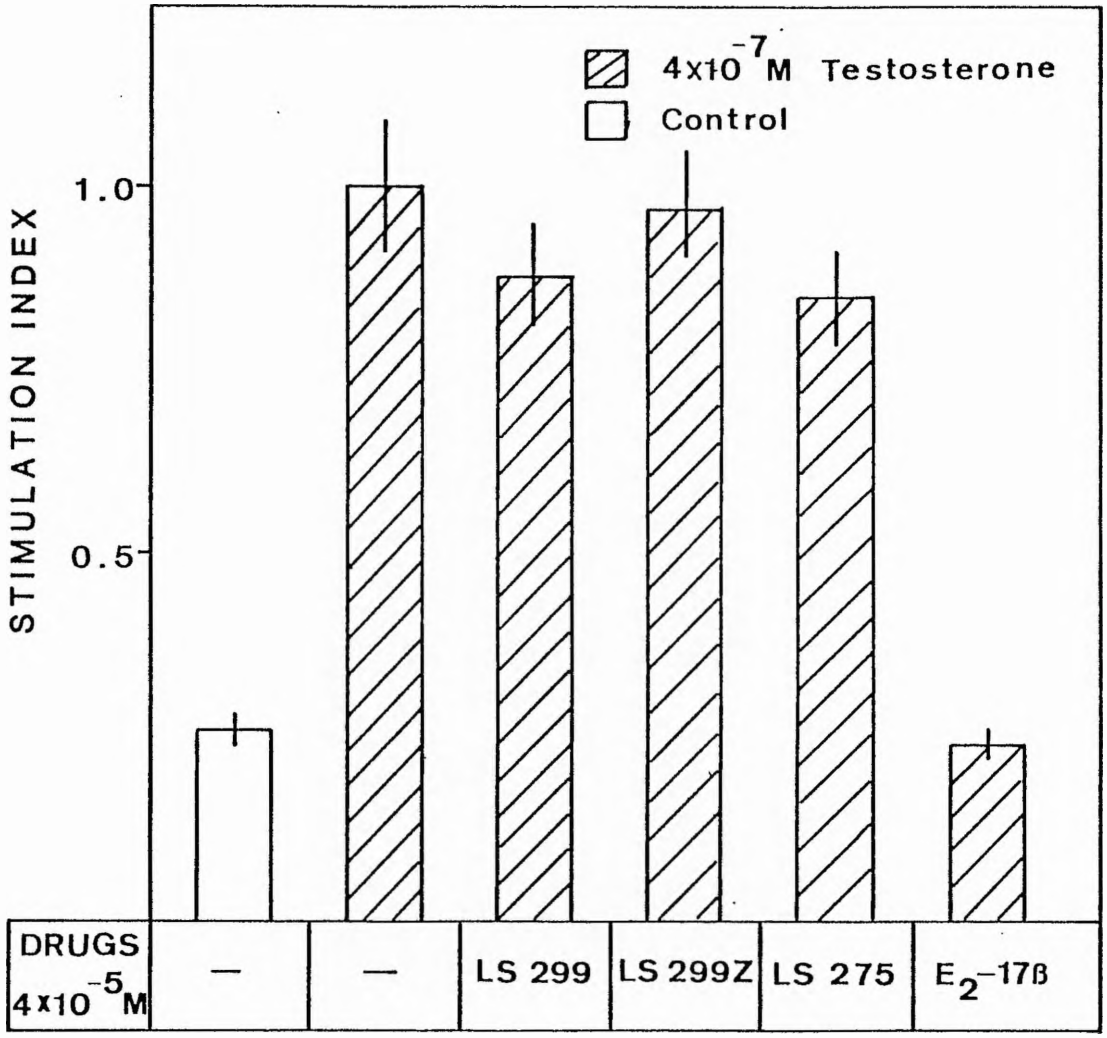

4x10⁻⁷M Testosterone
Control
STIMULATION INDEX
1.0
0.5
DRUGS
4x10⁻⁵M
—
—
LS 299
LS 299Z
LS 275
E2-17β

Fig. 3.21 Proliferative responses of rat ventral prostate on day 4 of organ culture in chemically-defined medium following treatment with 4 x 10^{-5}M estramustine phosphate sodium (LS 299Z), estramustine phosphate (LS 299), estramustine (LS 275) or oestradiol-17β (E_2-17β) during the first 48h and 4 x 10^{-7}M testosterone for the final 48h. 'Control' represents non-supplemented cultures.

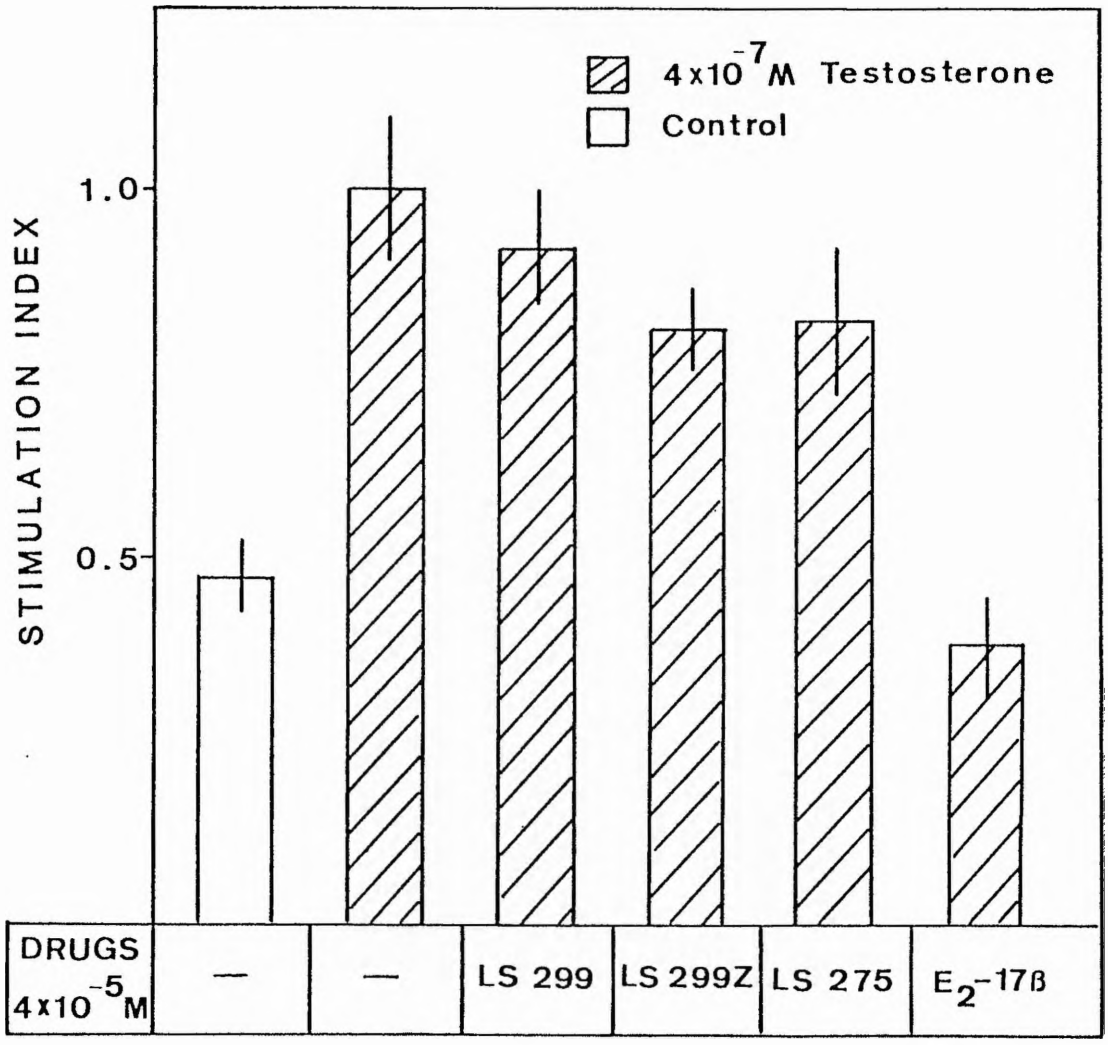

4x10⁻⁷M Testosterone
Control
STIMULATION INDEX
1.0
0.5
DRUGS
4x10⁻⁵M
—
—
LS 299
LS 299Z
LS 275
E2-17ß

Fig. 3.22 Effects of 4×10^{-9}, 4×10^{-7} and 4×10^{-5}M cyproterone acetate on the 4×10^{-9}M testosterone-stimulated response of rat ventral prostate on day 4 following organ culture in chemically-defined medium. Non-supplemented control cultures are represented by ■, medium only and □, medium containing the alcohol diluent.

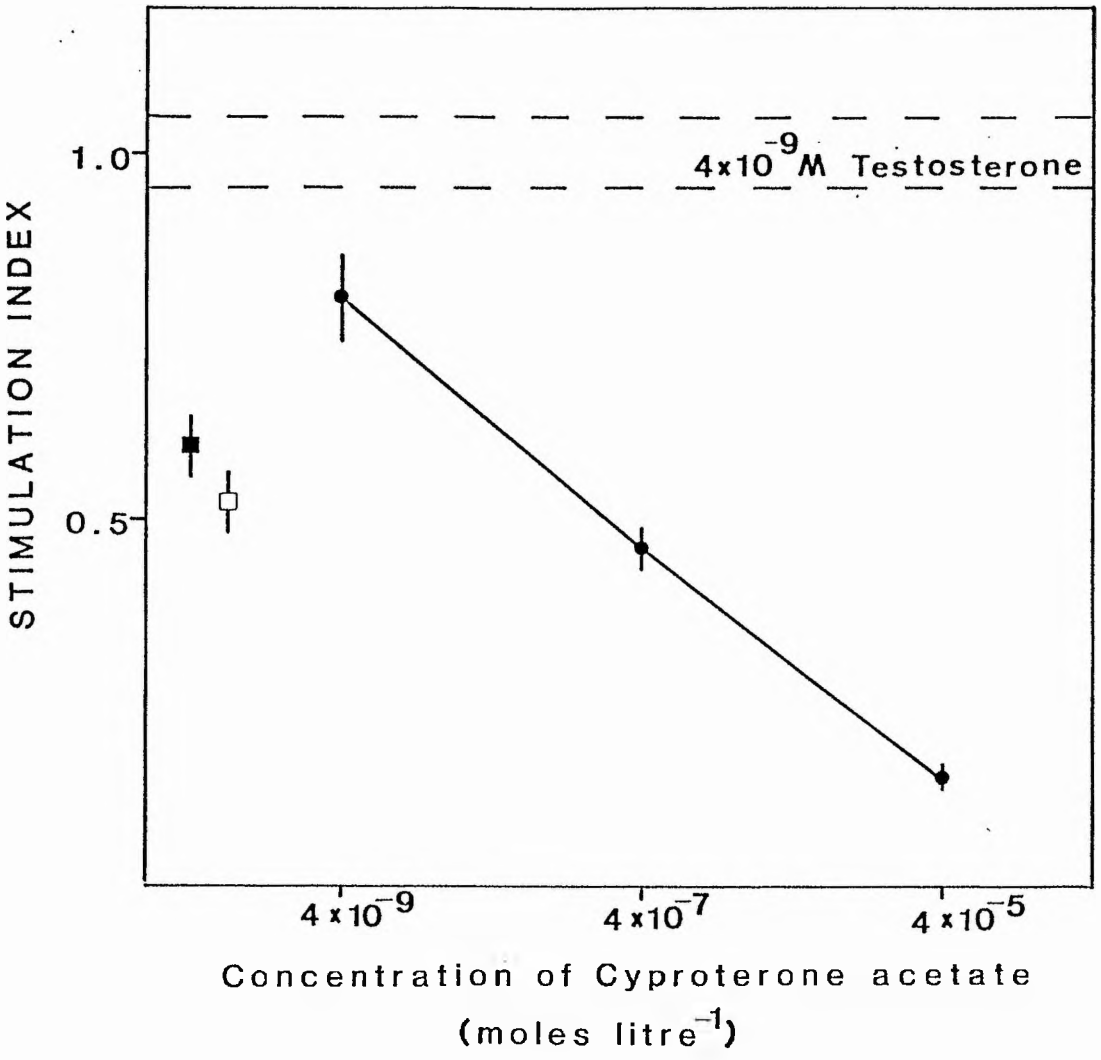

STIMULATION INDEX
1.0
0.5
4x10⁻⁹ M Testosterone
4 x 10⁻⁹
4 x 10⁻⁷
4 x 10⁻⁵
Concentration of Cyproterone acetate
(moles litre⁻¹)

Fig. 3.23 Effects of 4×10^{-9}, 4×10^{-7} and 4×10^{-5}M estramustine phosphate sodium (LS 299Z), estramustine phosphate (LS 299), estramustine (LS 275) and oestradiol-17β (E_2-17β) on the 4×10^{-9}M testosterone-stimulated response of rat ventral prostate on day 4 following organ culture in chemically-defined medium. Non-supplemented control cultures are represented by ■, medium only and □, medium containing the alcohol diluent.

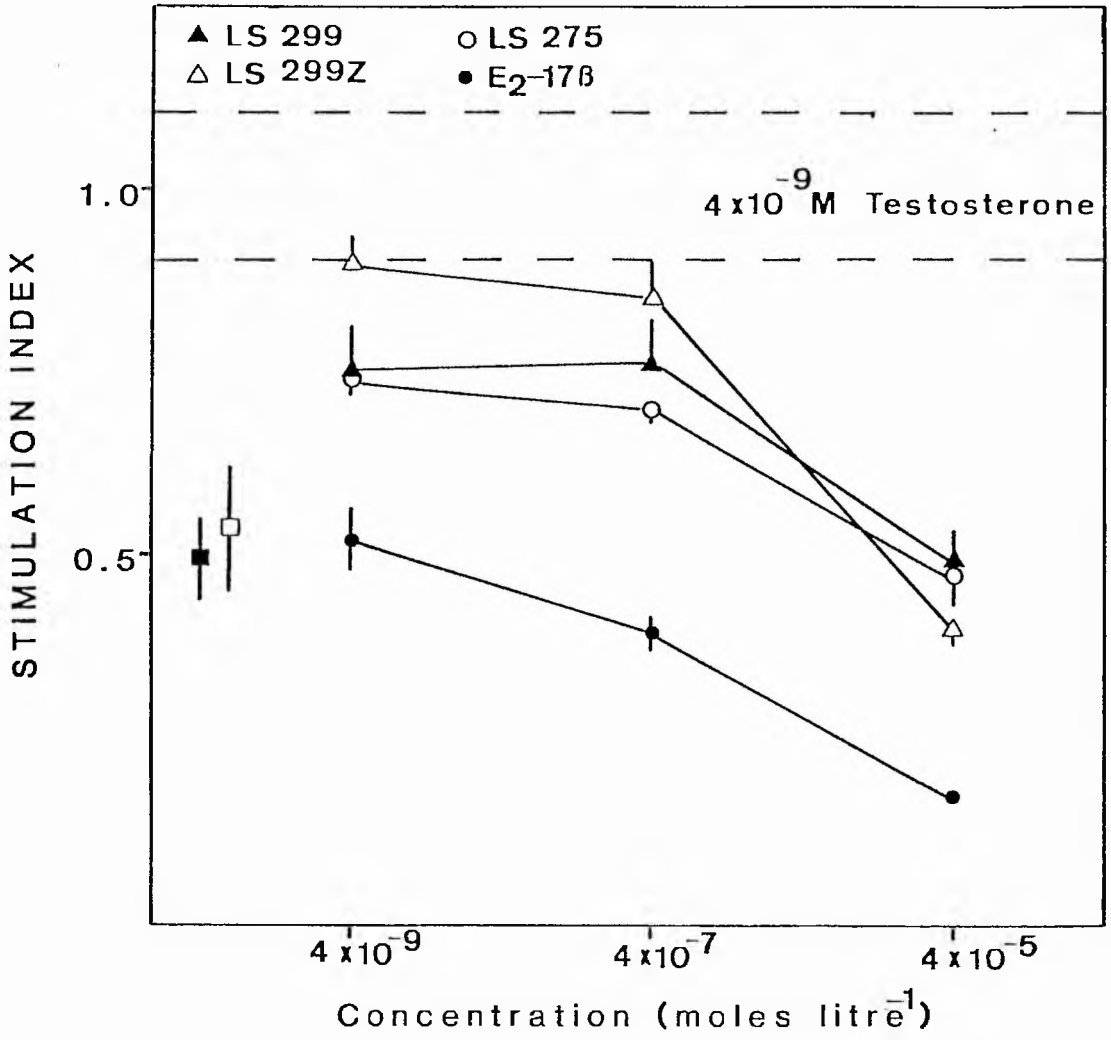

▲ LS 299
△ LS 299Z
○ LS 275
● E_2-17β
4×10^{-9} M Testosterone
1.0
0.5
STIMULATION INDEX
4×10^{-9}
4×10^{-7}
4×10^{-5}
Concentration (moles litre^{-1})

Fig. 3.24 Proliferative responses of rat ventral prostate on day 4 of organ culture in chemically-defined medium following treatment with 4 x 10^{-9}M testosterone for the first 48h and 4 x 10^{-5}M estramustine phosphate sodium (LS 299Z), estramustine phosphate (LS 299), estramustine (LS 275) or oestradiol-17β (E_2-17β) during the final 48h. 'Control' represents non-supplemented cultures.

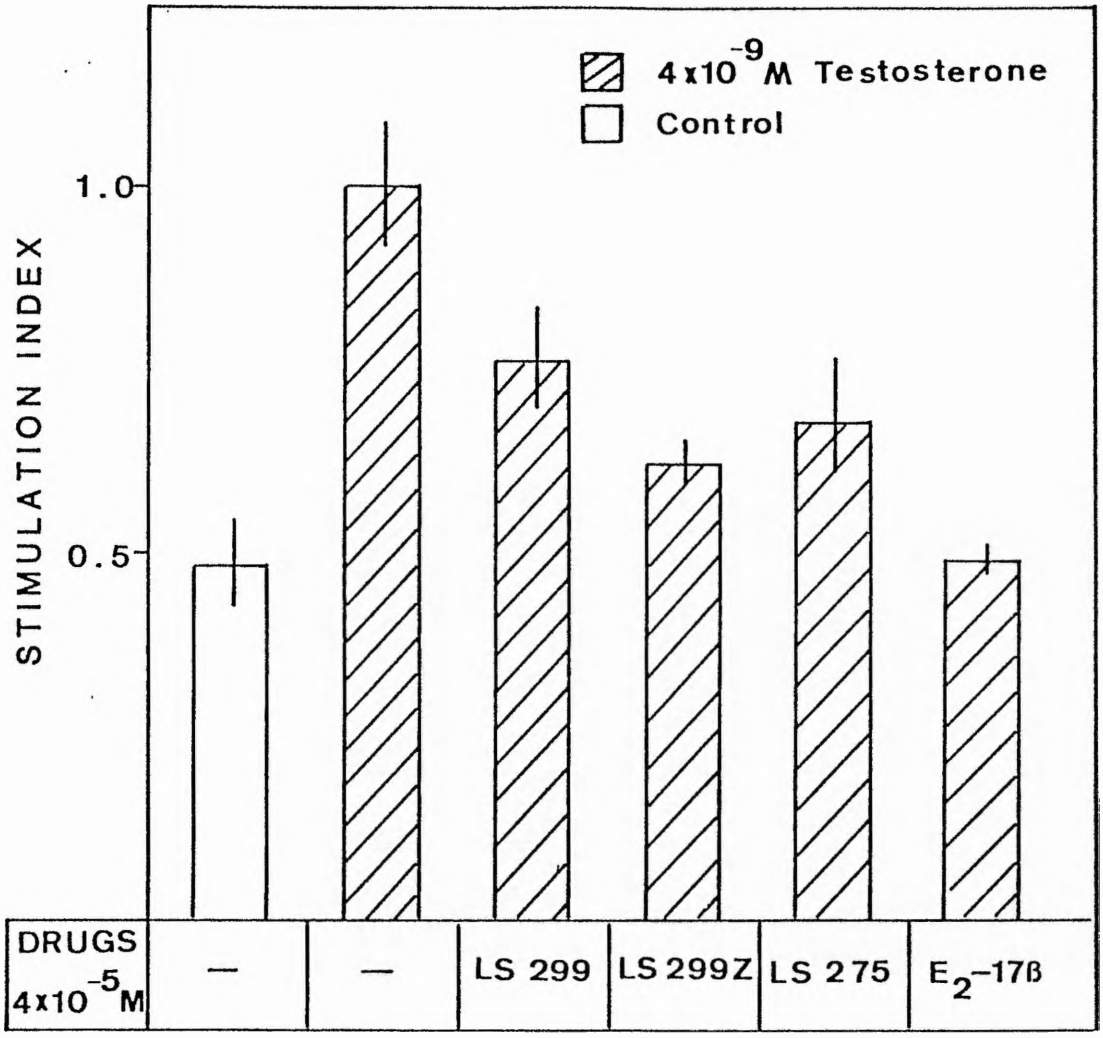

4x10⁻⁹M Testosterone
Control
STIMULATION INDEX
1.0
0.5
DRUGS
4x10⁻⁵M
—
—
LS 299
LS 299Z
LS 275
E2-17β

Fig. 3.25 Proliferative responses of rat ventral prostate on day 4 of organ culture in chemically-defined medium following treatment with 4 x 10^{-5}M estramustine phosphate sodium (LS 299Z), estramustine phosphate (LS 299), estramustine (LS 275) or oestradiol-17β (E_2-17β) for the first 48h and 4 x 10^{-9}M testosterone during the final 48h. 'Control' represents non-supplemented cultures.

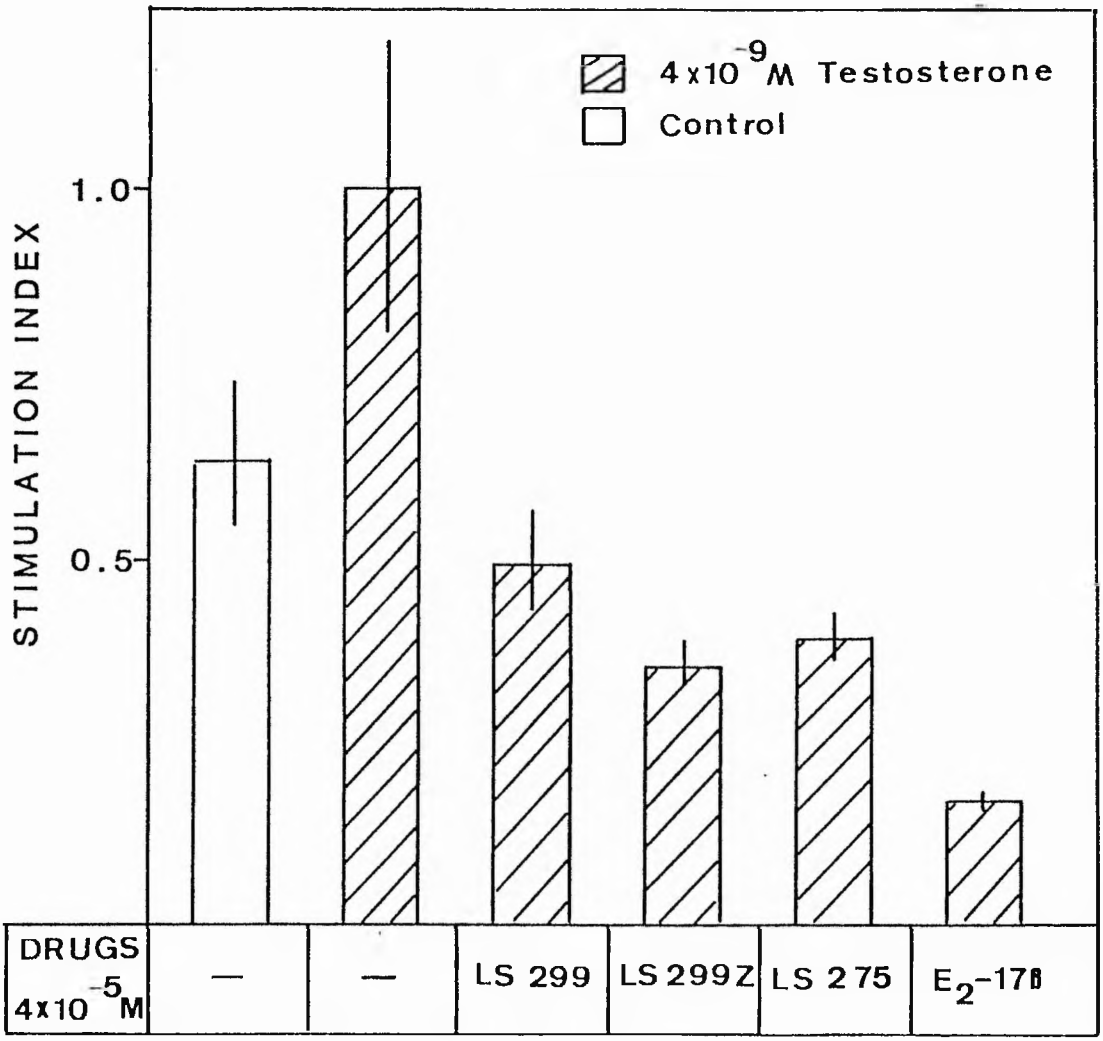

4x10⁻⁹M Testosterone
Control
STIMULATION INDEX
1.0
0.5
DRUGS
4x10⁻⁵M
—
—
LS 299
LS 299Z
LS 275
E2-17β

Fig. 3.26 Effects of 4×10^{-5}M estramustine phosphate (LS 299), estramustine (LS 275) and oestradiol-17β (E_2-17β) on the 4×10^{-6}M testosterone-stimulated response of rat ventral prostate on day 4 of organ culture in the absence and presence of 5% foetal calf serum (FCS).

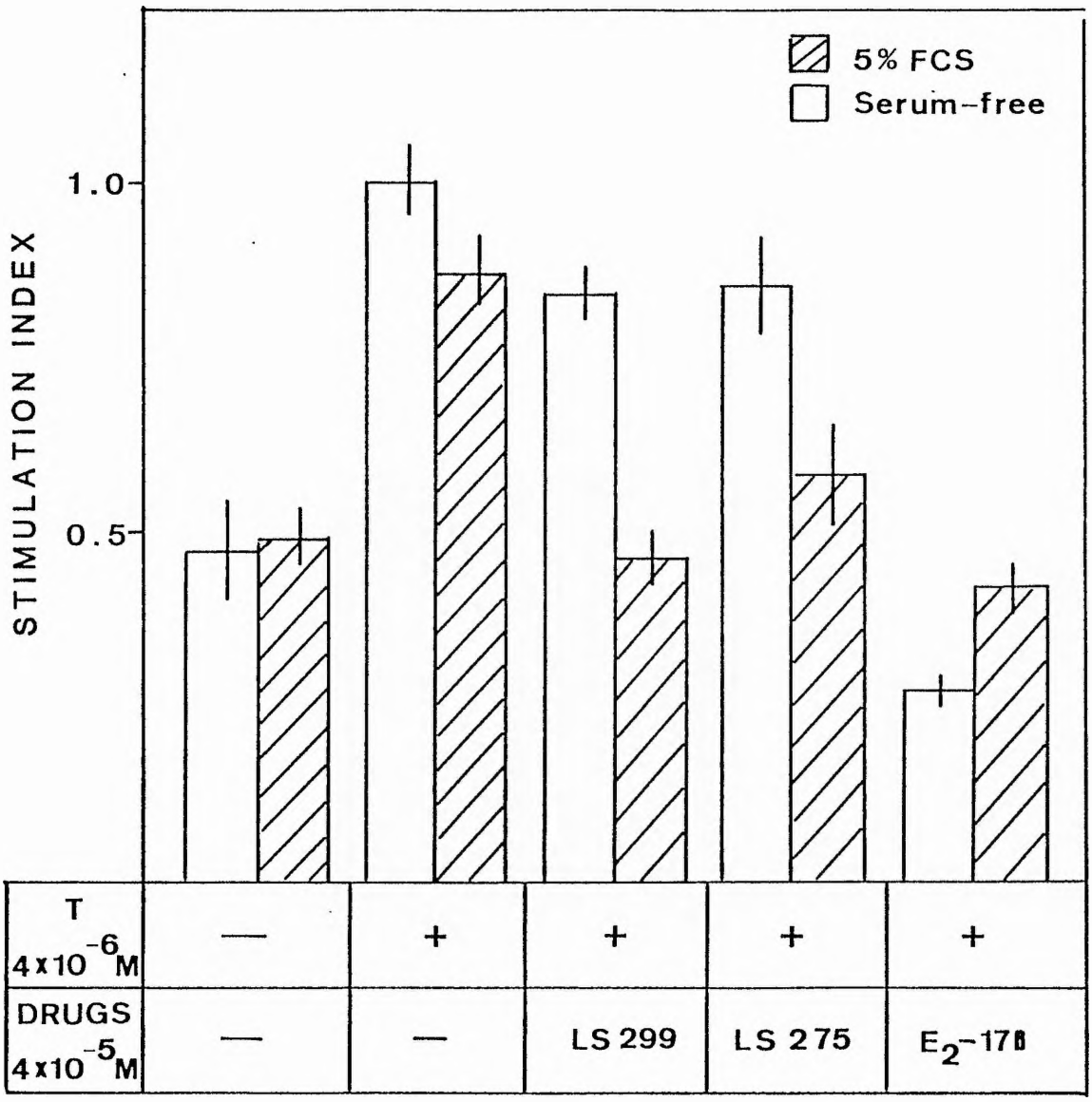

5% FCS
Serum-free
STIMULATION INDEX
1.0
0.5
T
4x10⁻⁶M
DRUGS
4x10⁻⁵M
—
+
+
+
+
—
—
LS 299
LS 275
E2-17β

Fig. 3.27 Effects of 4 x 10^{-5}M estramustine phosphate (LS 299), estramustine (LS 275) and oestradiol-17β (E_2-17β) on the 4 x 10^{-6}M testosterone-stimulated response of rat ventral prostate on day 4 of organ culture in the absence and presence of foetal calf serum (5, 10 and 20%).

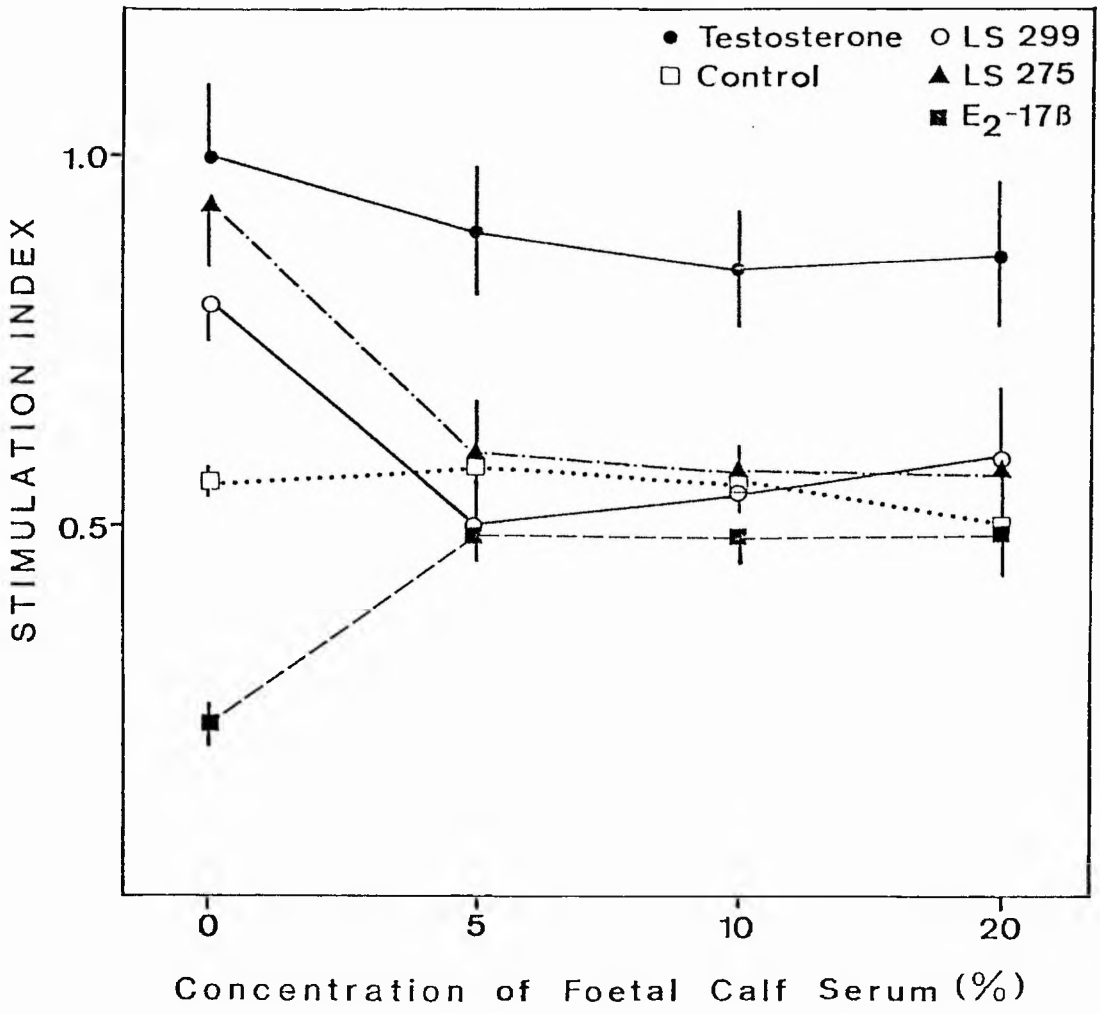
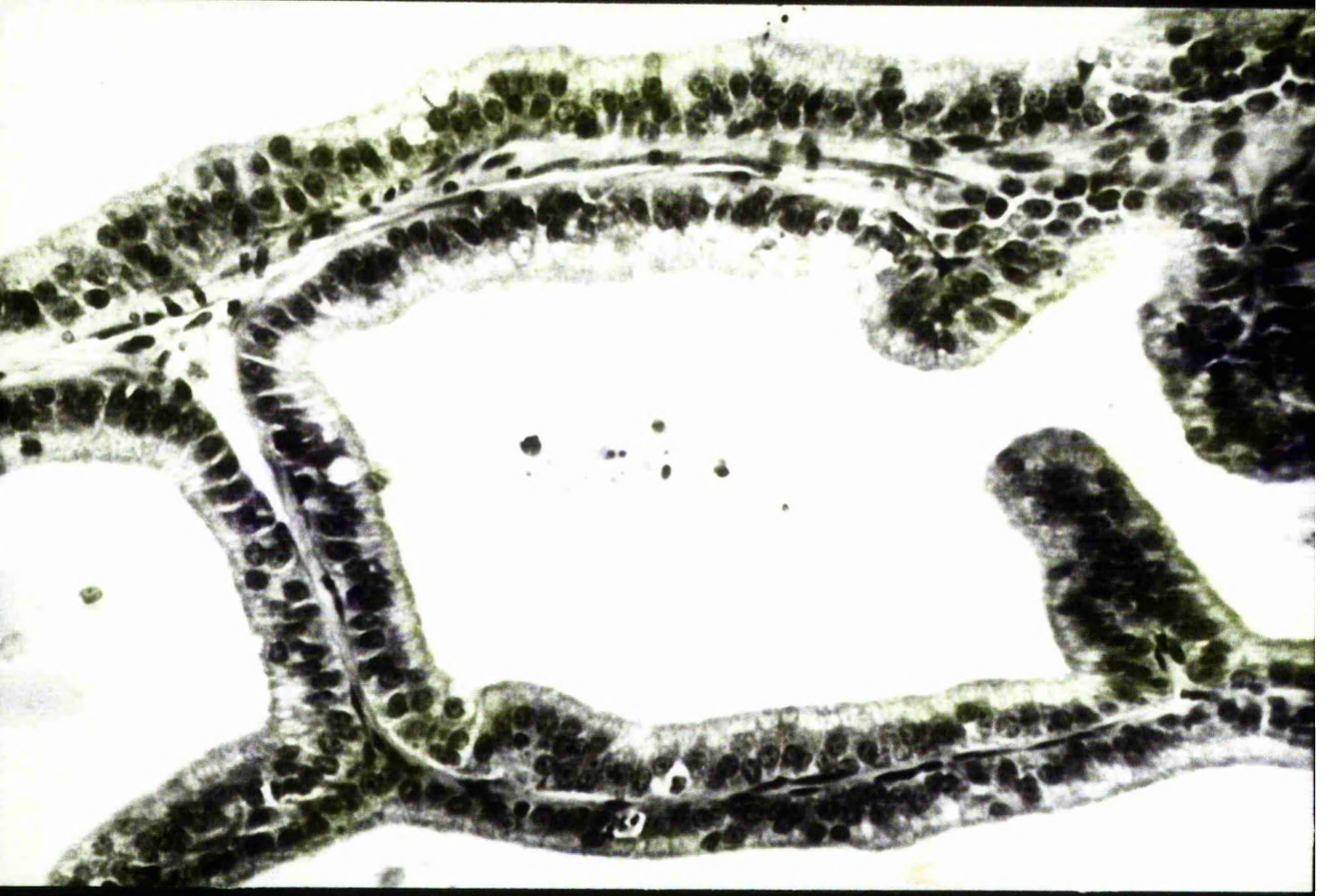

● Testosterone
○ LS 299
□ Control
▲ LS 275
■ E_2-17β
STIMULATION INDEX
1.0
0.5
0
5
10
20
Concentration of Foetal Calf Serum (%)

Plate 3.15 Alveoli in an explant of young adult rat ventral prostate cultured for four days in chemically-defined medium supplemented with 4×10^{-7}M testosterone, showing well-maintained columnar epithelium and areas of epithelial cell proliferation. Haematoxylin & Eosin. X400.

Plate 3.16 Alveoli in a similar explant cultured for four days in chemically-defined medium supplemented with 4×10^{-7}M testosterone and 4×10^{-7}M cyproterone acetate, showing close resemblance to the testosterone control culture (Plate 3.15). Haematoxylin & Eosin. X400.



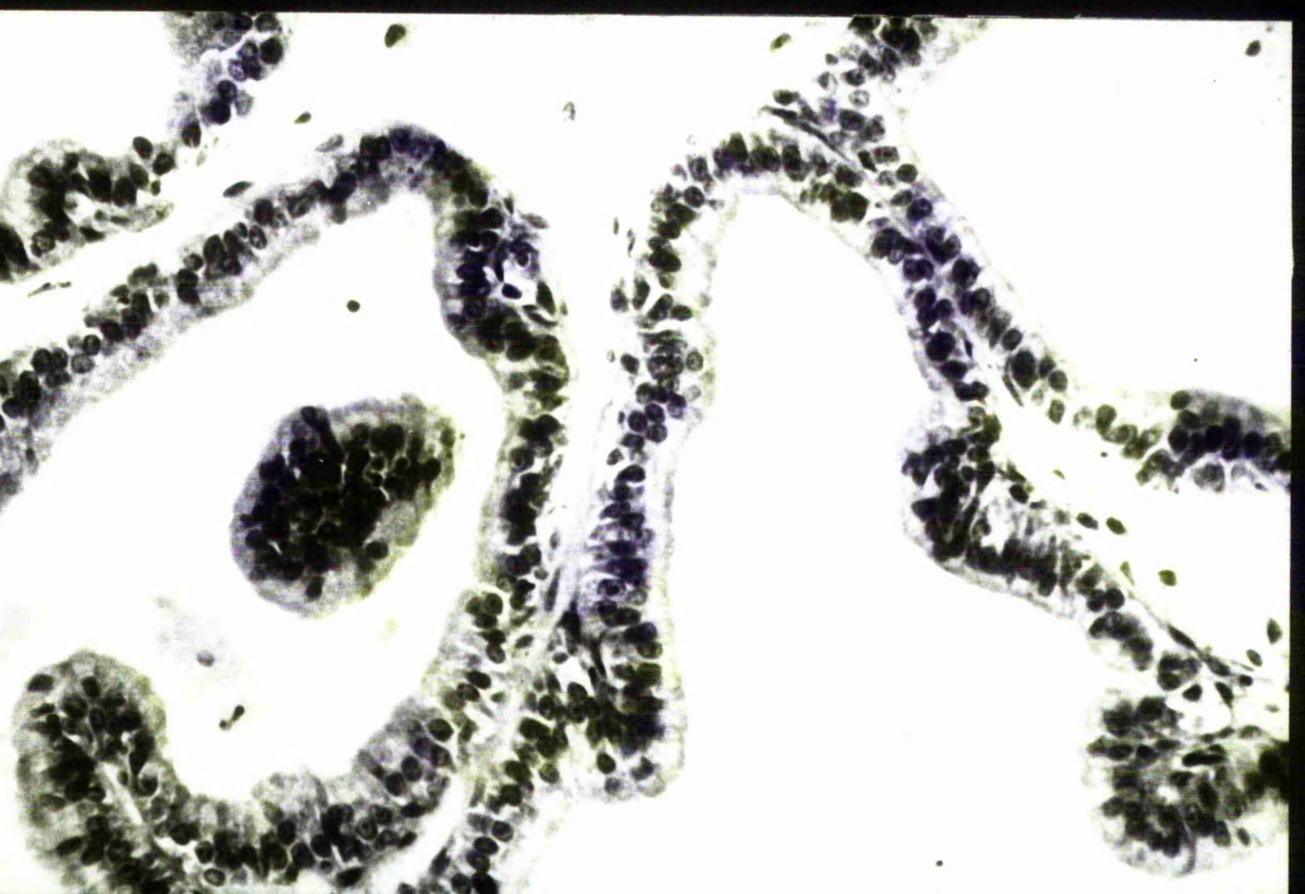
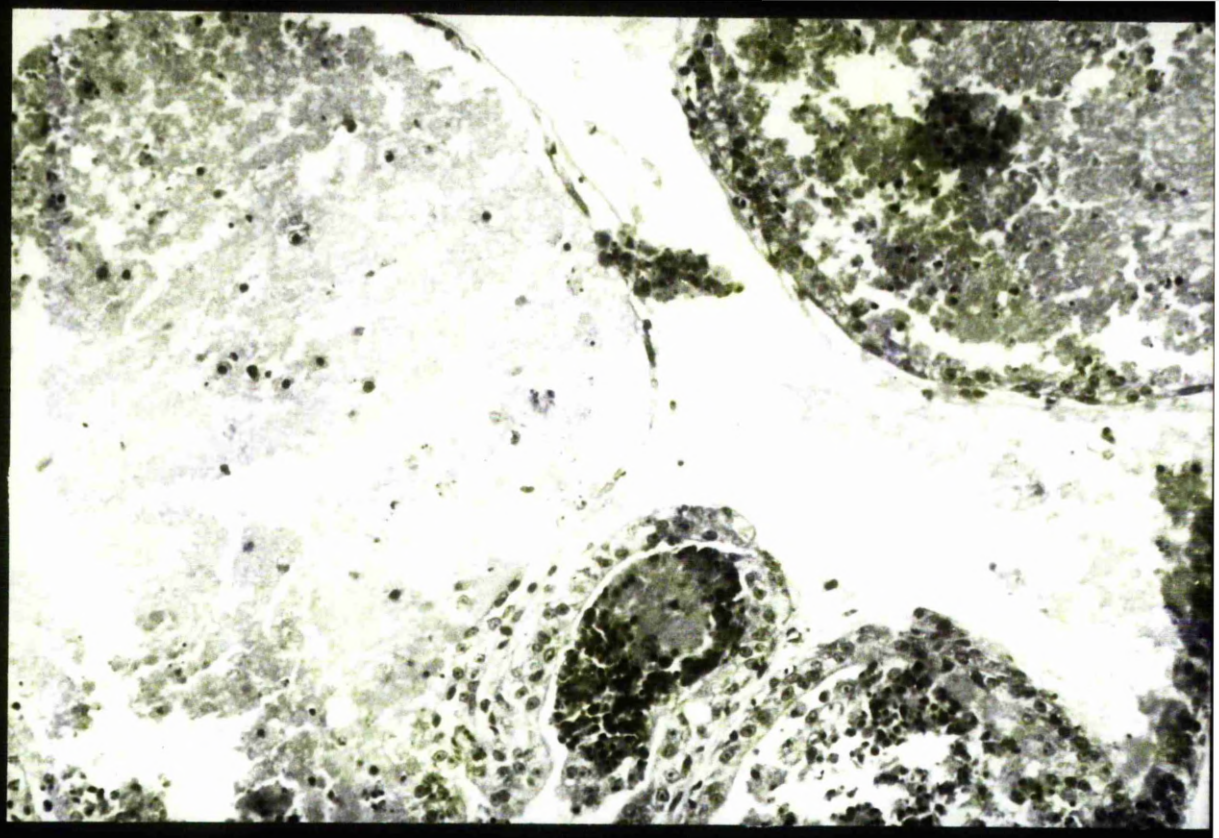


Plate 3.17 Alveoli in a similar explant sultured for four days in chemically-defined medium supplemented with 4×10^{-7}M testosterone and 4×10^{-5}M cyproterone acetate, showing widespread necrosis of the alveolar epithelium and fibromuscular stroma. Haematoxylin & Eosin. X200.

Plate 3.18 Alveoli in an explant of young adult rat ventral prostate cultured for four days in chemically-defined medium supplemented with 4×10^{-7}M testosterone, showing well-maintained, actively secreting columnar epithelium and evidence of epithelial cell proliferation. Haematoxylin & Eosin. X400.



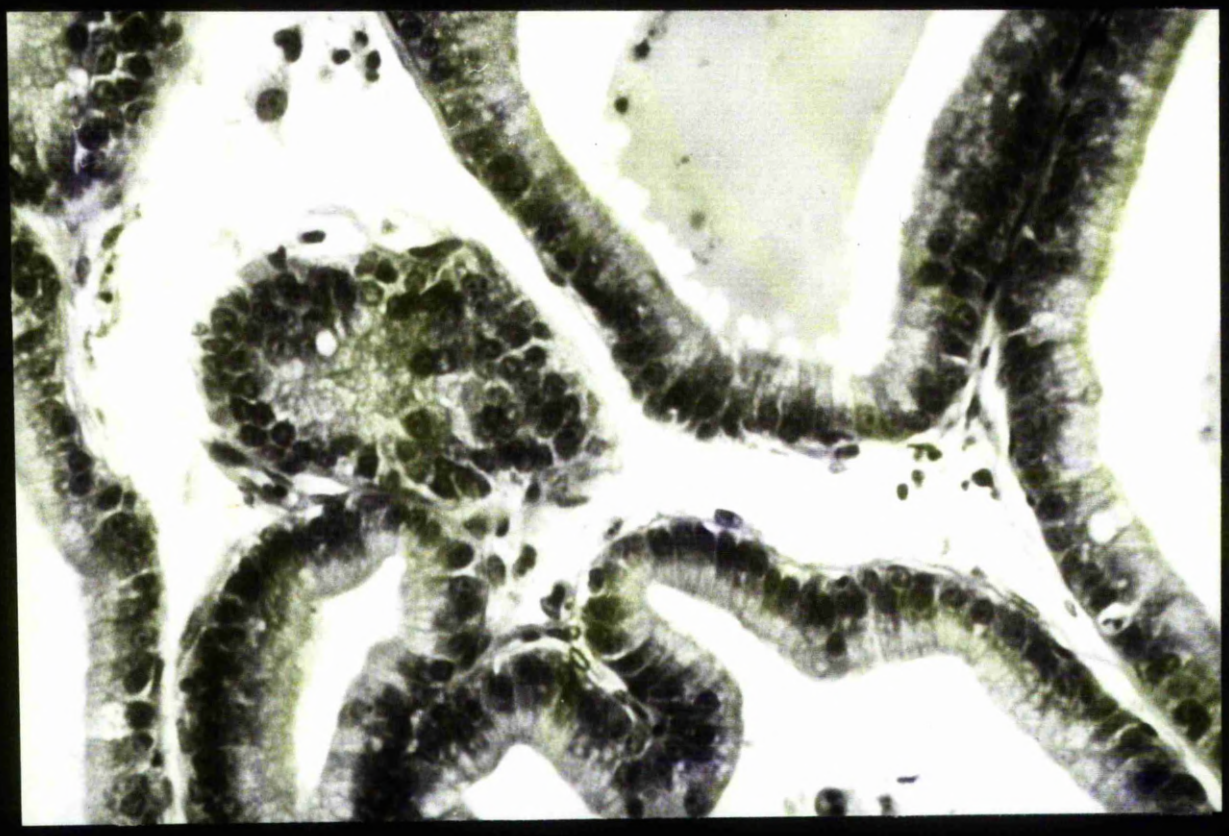


Plate 3.19 Alveoli in a similar explant cultured for four days in chemically-defined medium supplemented with 4 x 10^{-7}M testosterone and 4 x 10^{-5}M estramustine phosphate (LS 299), showing the same well-preserved columnar epithelium as seen in the testosterone control culture (Plate 3.18). Haematoxylin & Eosin. X400.

Plate 3.20 Alveoli in a similar explant cultured for four days in chemically-defined medium supplemented with 4 x 10^{-7}M testosterone and 4 x 10^{-5}M estramustine (LS 275), also showing close resemblance to the testosterone control culture (Plate 3.18). Haematoxylin & Eosin. X400.

Plate 3.21 Alveoli in a similar explant cultured for four days in chemically-defined medium supplemented with 4×10^{-7}M testosterone and 4×10^{-5}M oestradiol-17β, showing extensive necrosis of the alveolar epithelium and fibromuscular stroma. Haematoxylin & Eosin. X200.

Plate 3.22 Alveoli in an explant of young adult rat ventral prostate cultured for four days in chemically-defined medium supplemented with 4×10^{-9}M testosterone, showing well-maintained columnar epithelium and evidence of epithelial cell proliferation. Haematoxylin & Eosin. X400.

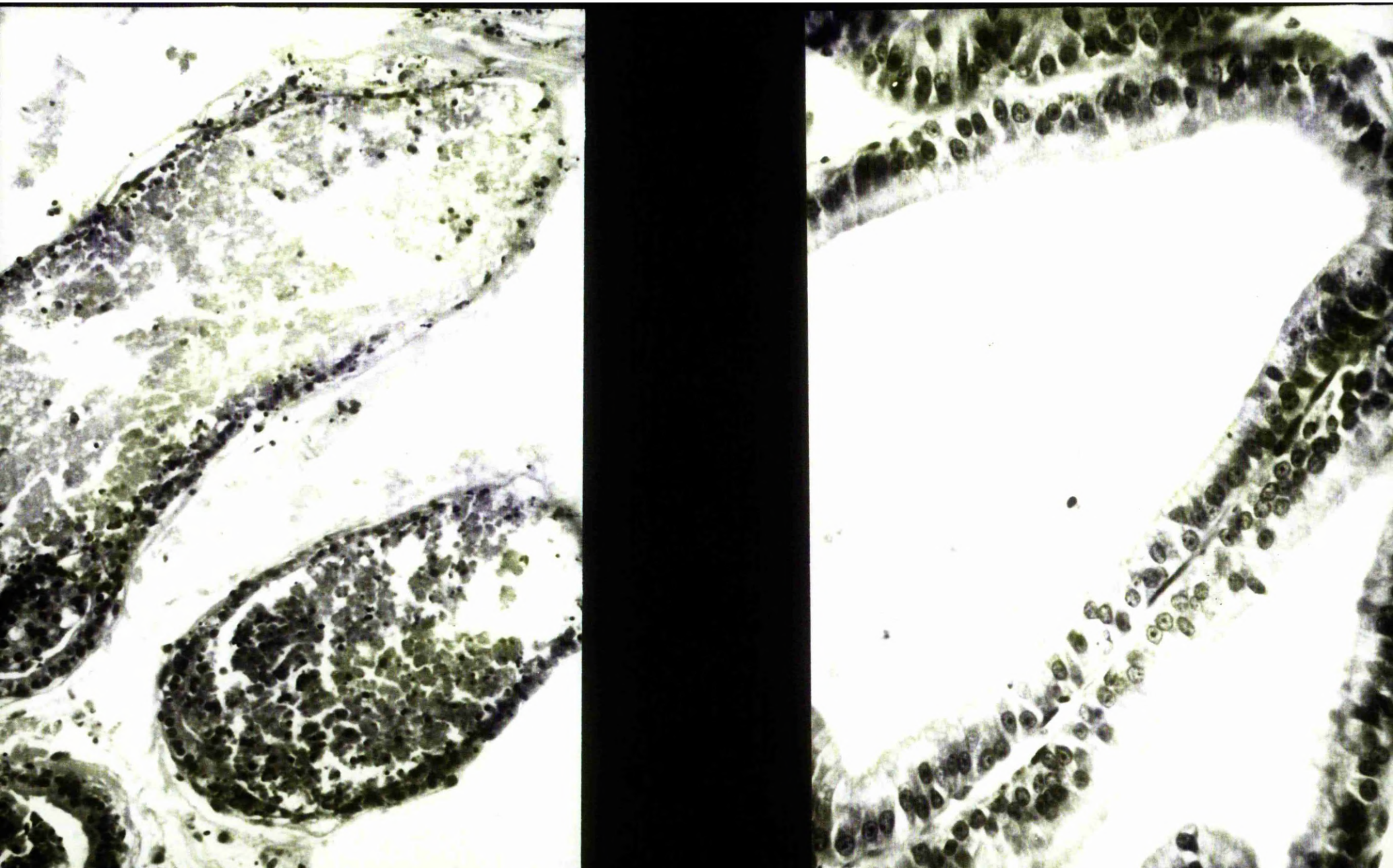


Plate 3.23 Alveoli in a similar explant cultured for four days in chemically-defined medium supplemented with 4×10^{-9}M testosterone and 4×10^{-7}M cyproterone acetate, showing reduced epithelial height and lack of fresh secretion, typical of androgen-deprived cultures. Haematoxylin & Eosin. X400.

Plate 3.24 Alveoli in an explant of young adult rat ventral prostate cultured for four days in chemically-defined medium supplemented with 4×10^{-9}M testosterone, showing well-preserved columnar epithelium and areas of epithelial hyperplasia. Haematoxylin & Eosin. X400.

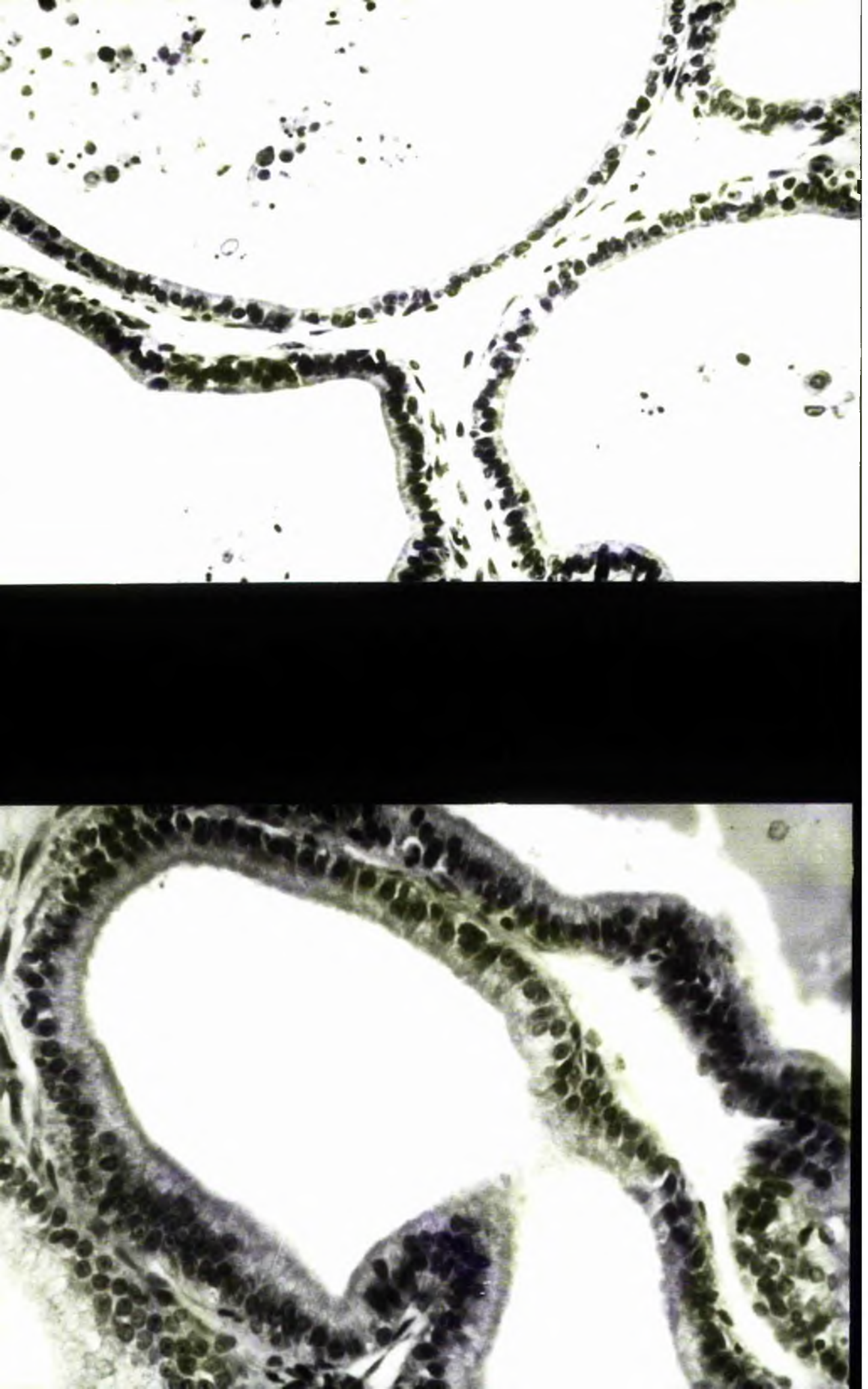


Plate 3.25 Alveoli in a similar explant cultured for four days in chemically-defined medium supplemented with 4×10^{-9}M testosterone and 4×10^{-5}M estramustine phosphate (LS 299), showing low cuboidal epithelium and reduced secretory activity, typical of androgen-deprived cultures. Haematoxylin & Eosin. X400.

Plate 3.26 Alveoli in a similar explant cultured for four days in chemically-defined medium supplemented with 4×10^{-9}M testosterone and 4×10^{-7}M oestradiol-17β, showing atrophic alveolar epithelium and necrosis of the fibromuscular stroma. Haematoxylin & Eosin. X200.

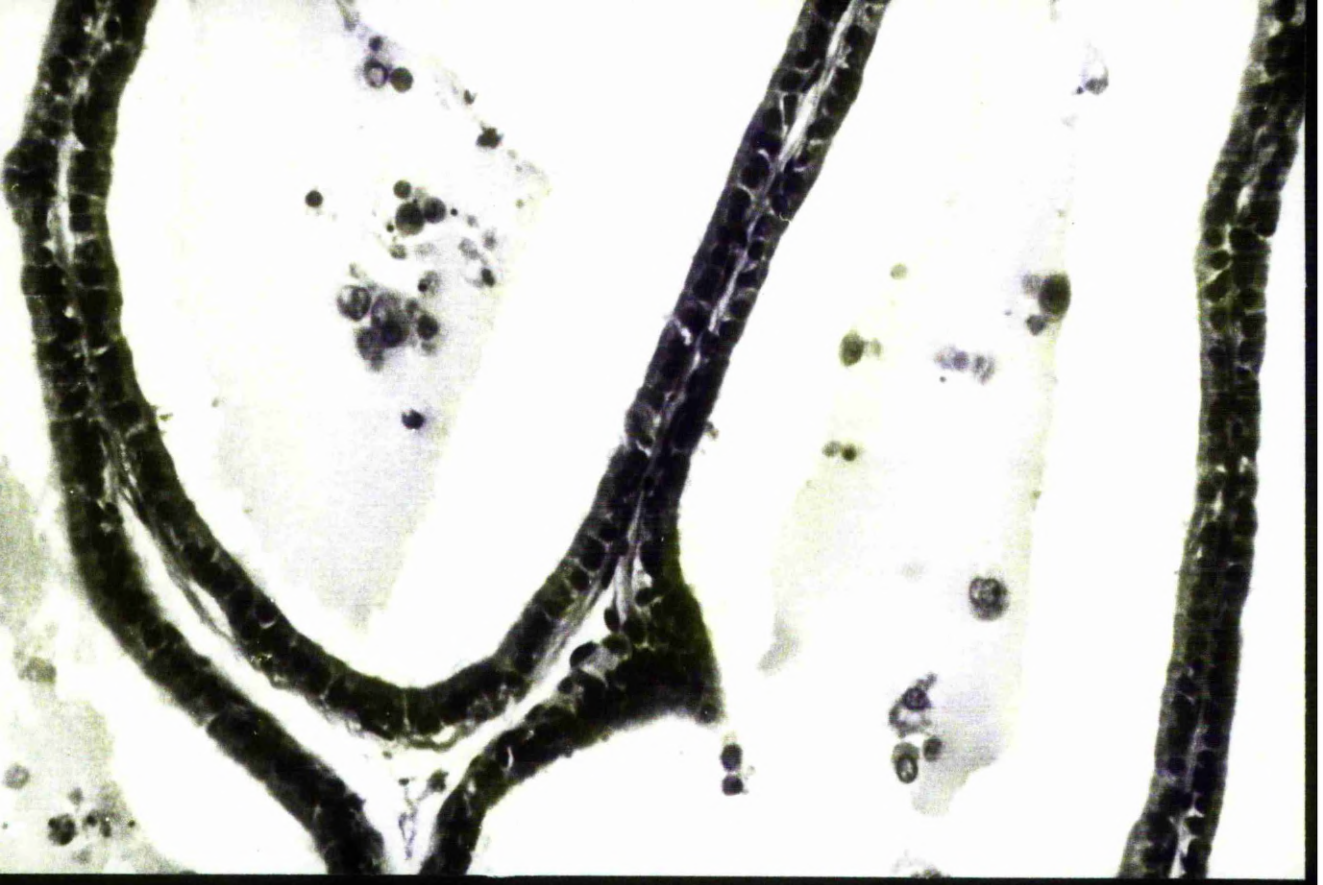


Plate 3.27 Alveoli in an explant of young adult rat ventral prostate cultured for four days in chemically-defined medium supplemented with 4×10^{-6}M testosterone, showing well-maintained, actively secreting columnar epithelium. Haematoxylin & Eosin. X400.

Plate 3.28 Alveoli in a similar explant cultured for four days in chemically-defined medium supplemented with 4×10^{-6}M testosterone and 4×10^{-5}M estramustine phosphate (LS 299), showing close resemblance to the testosterone control culture (Plate 3.27). Haematoxylin & Eosin. X400.

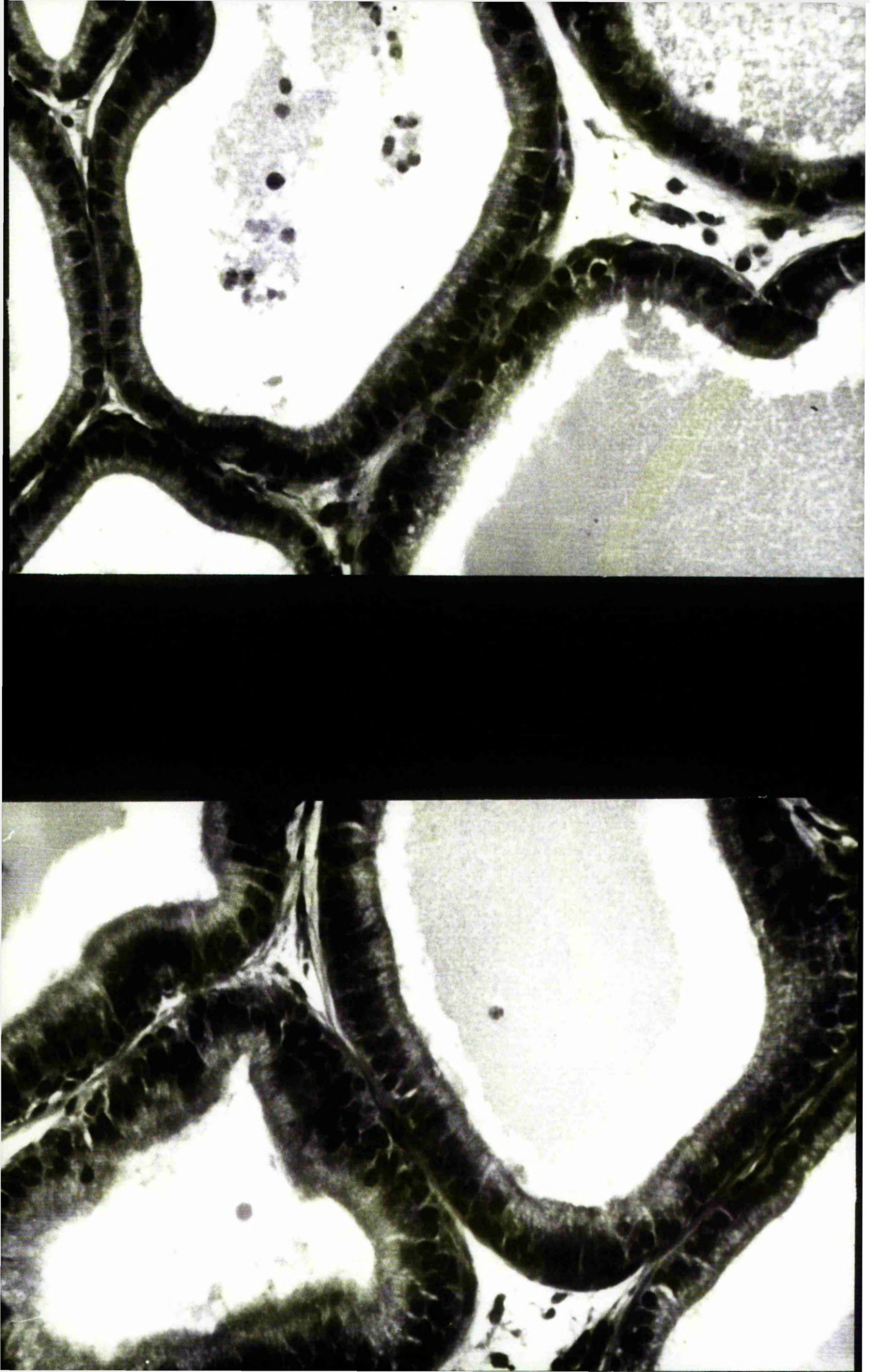
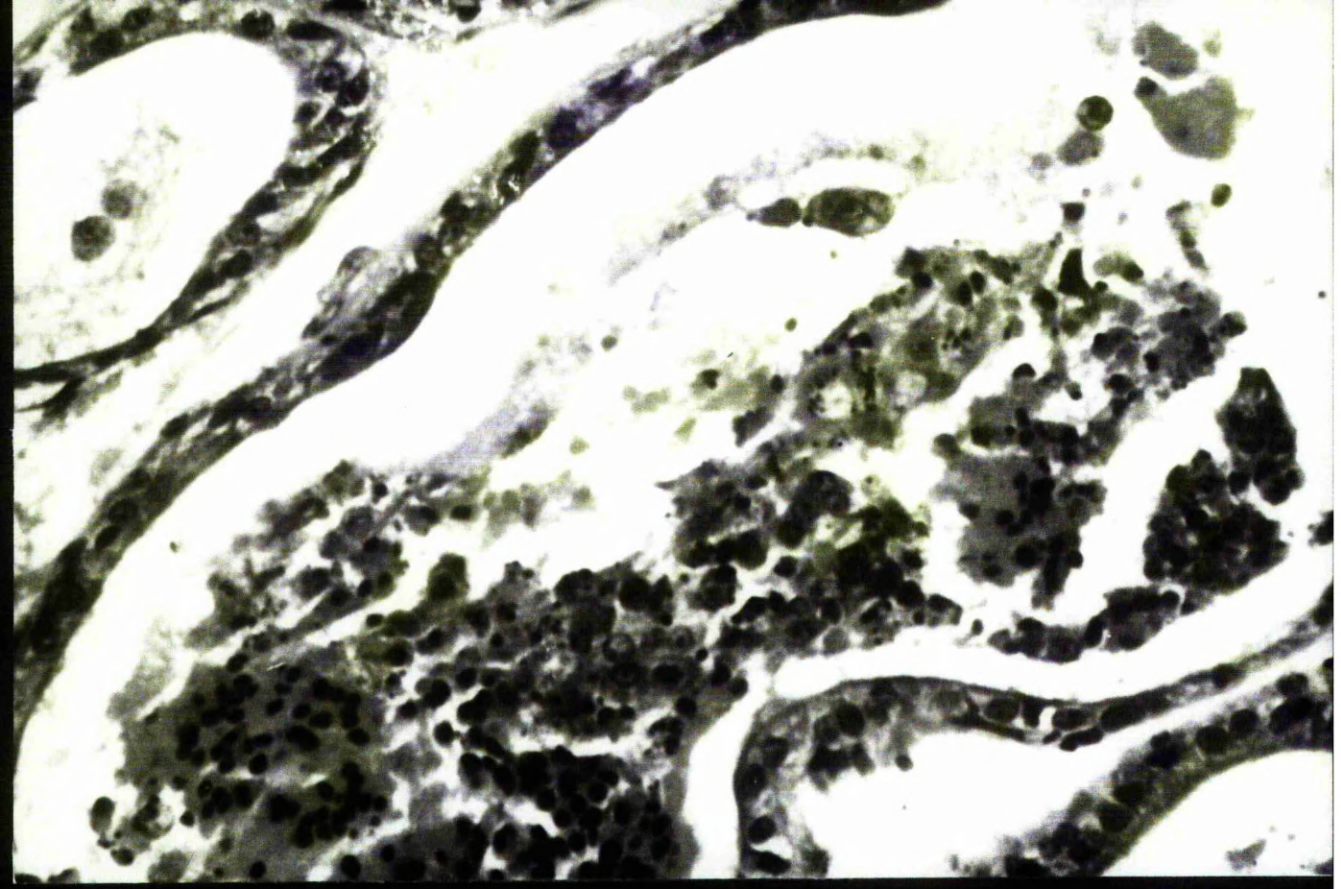


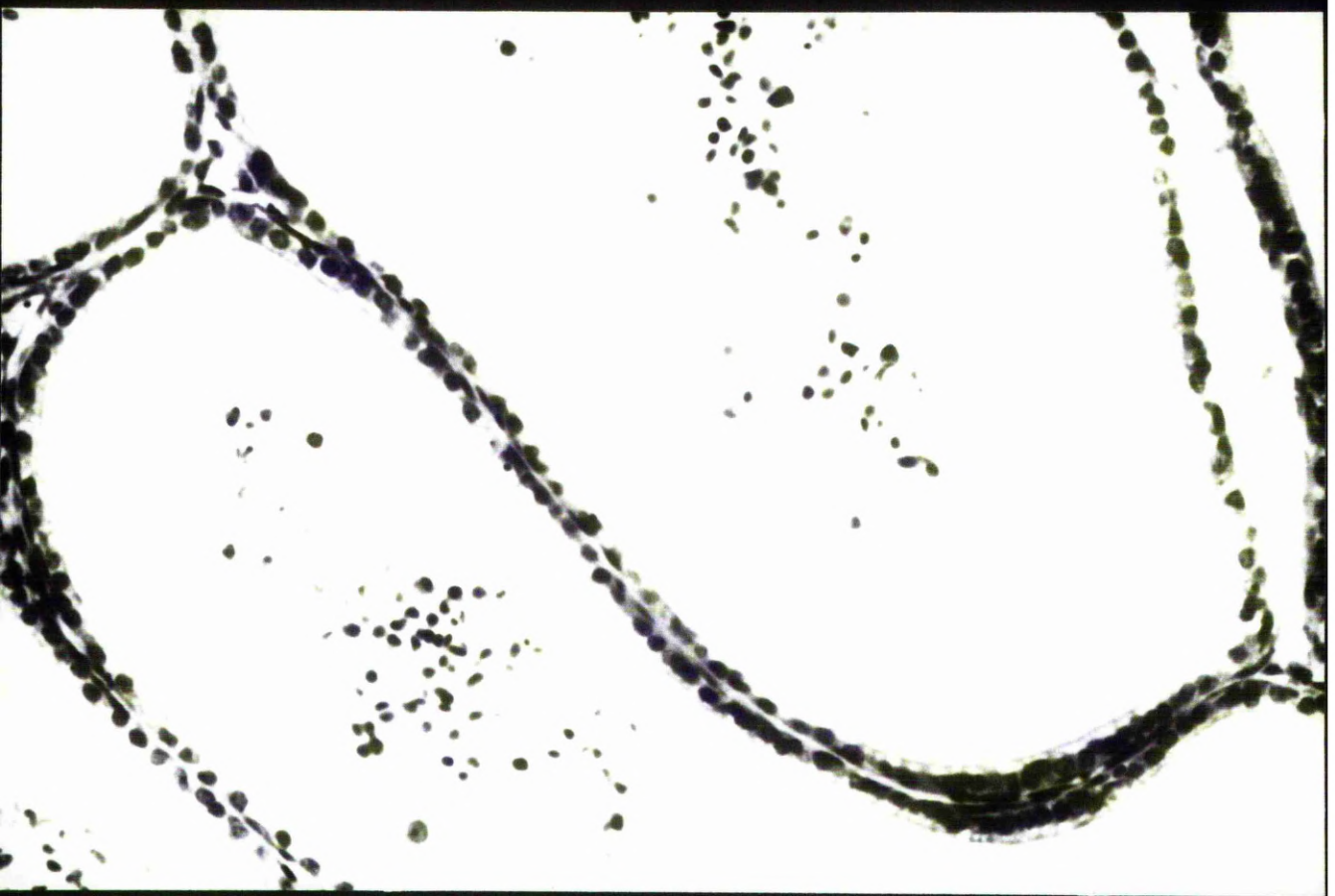
Plate 3.29 Alveoli in a similar explant cultured for four days in chemically-defined medium supplemented with 4×10^{-6}M testosterone and 4×10^{-5}M oestradiol-17β, showing extensive necrosis of the epithelium and stromal tissue. Haematoxylin & Eosin. X200.

Plate 3.30 Alveoli in an explant of young adult rat ventral prostate cultured for four days in semi-defined medium (5% foetal calf serum) supplemented with 4×10^{-6}M testosterone, showing well-maintained, actively secreting columnar epithelium. Haematoxylin & Eosin. X400.

Plate 3.31 Alveoli in a similar explant cultured for four days in semi-defined medium (5% foetal calf serum) supplemented with 4 x 10^{-6}M testosterone and 4 x 10^{-5}M estramustine phosphate (LS 299), showing marked degeneration and necrosis of the alveolar epithelium. Haematoxylin & Eosin. X400.

Plate 3.32 Alveoli in a similar explant cultured for four days in semi-defined medium (5% foetal calf serum) supplemented with 4 x 10^{-6}M testosterone and 4 x 10^{-5}M oestradiol-17β, showing severe atrophy of the alveolar epithelium and absence of secretory activity. Haematoxylin & Eosin. X400.





3.3 HISTOCHEMICAL DEMONSTRATION OF ACID PHOSPHATASE ACTIVITY IN HUMAN BENIGN PROSTATIC HYPERPLASIA (BPH) AND RAT VENTRAL PROSTATE

Acid phosphatase activity in fresh-frozen sections of human BPH was localized in the alveolar epithelium and stained bright red when using the azo-dye, Fast Garnet GBC (Plate 3.33). The intensity of the reaction was similar when the cryostat sections were air-dried without fixation or were fixed for 15 minutes in either cold acetone or 10% neutral formalin. Following incubation in 10% neutral formalin for 24h the acid phosphatase reaction remained positive, but the intensity of the reaction was reduced (Plate 3.34).

Acid phosphatase activity in cryostat sections of immature (4 weeks old) and young adult (4 to 6 months old) rat ventral prostate was localized in the alveolar epithelium but, unlike human BPH, the reaction was much less intense (Plate 3.35). Sections air-dried without fixation or fixed in either cold acetone or 10% neutral formalin for 15 minutes all exhibited reactions of similar intensity. However, after incubation in 10% neutral formalin for 5h, acid phosphatase activity was no longer demonstrable in prostatic tissue from either immature or young adult rats (Plate 3.36). Similar results were observed in cryostat sections of liver and kidney obtained from young adult rats.

Unlike young adult rat ventral prostate, acid phosphatase activity in cryostat sections of ventral prostatic tissue from retired breeding rats (> 12 months old) was localized in discrete clumps throughout the alveolar epithelium (Plate 3.37). The distribution and

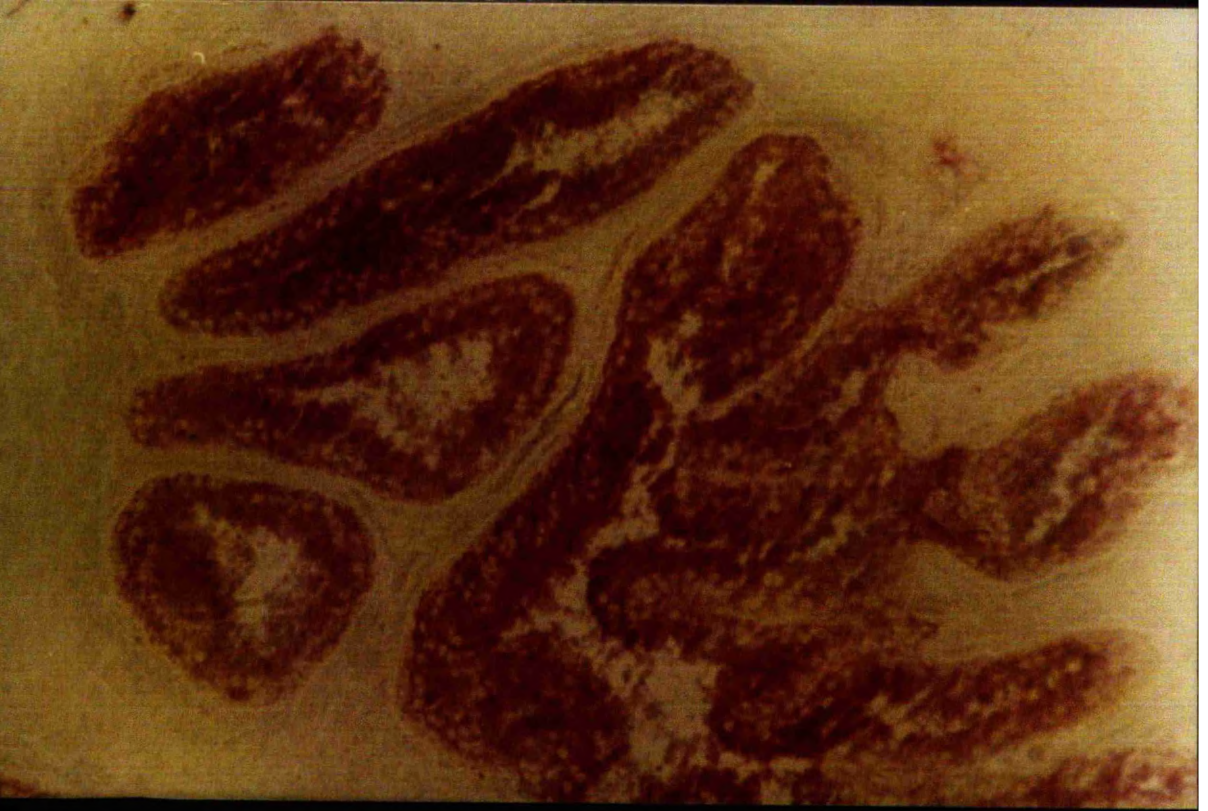
intensity of the reaction were similar whether the sections were air-dried without fixation or fixed for 15 minutes in either cold acetone or 10% neutral formalin. Furthermore, acid phosphatase activity in aged rat ventral prostate persisted following 5h of formalin treatment and the intensity of the reaction was not altered (Plate 3.38).

Control sections incubated in either the substrate (napthol AS-MX phosphate) or dye (Fast Garnet GBC) alone did not exhibit any staining reaction.

Plates 3.33 - 3.38 are cryostat sections of fresh biopsy specimens of human BPH and rat ventral prostate stained for acid phosphatase in a solution of Napthol AS-MX phosphate and Fast Garnet GBC diazonium salt for 60 minutes at room temperature. Mayer's haemalum was used as a nuclear counterstain.

Plate 3.33 Human BPH showing intense acid phosphatase staining in the alveolar epithelium. Acetone fixation. X160.

Plate 3.34 Section of the same BPH specimen showing a positive, but reduced, reaction for acid phosphatase following fixation in 10% neutral formalin for 24h. X160.



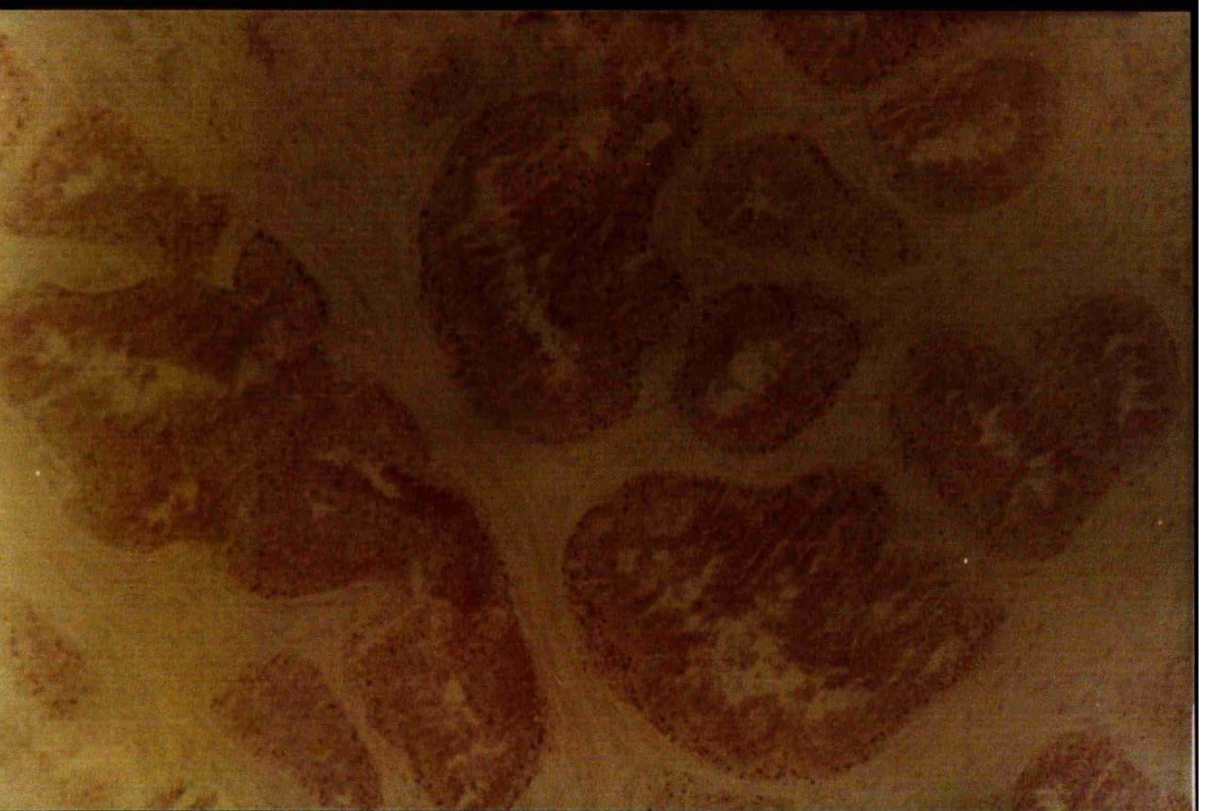


Plate 3.35 Young adult rat ventral prostate showing acid phosphatase staining in the alveolar epithelium. Acetone fixation. X160.

Plate 3.36 Section from the same specimen of rat ventral prostate showing the absence of acid phosphatase activity after fixation in 10% neutral formalin for 5h. X160.

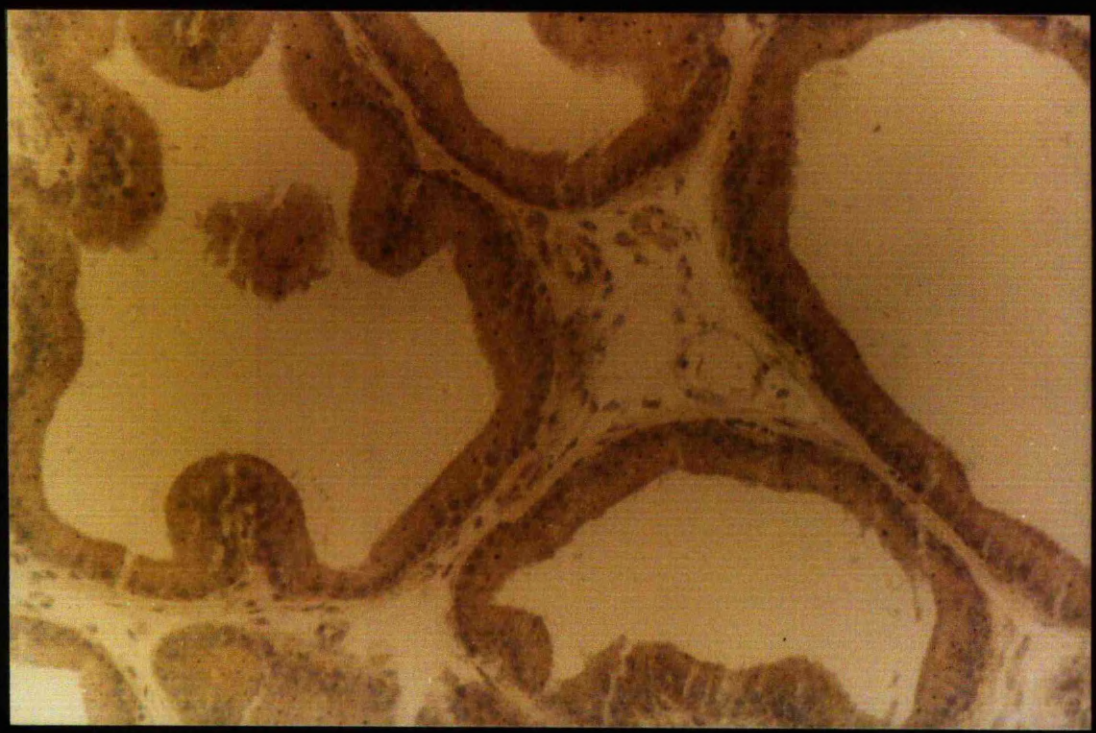
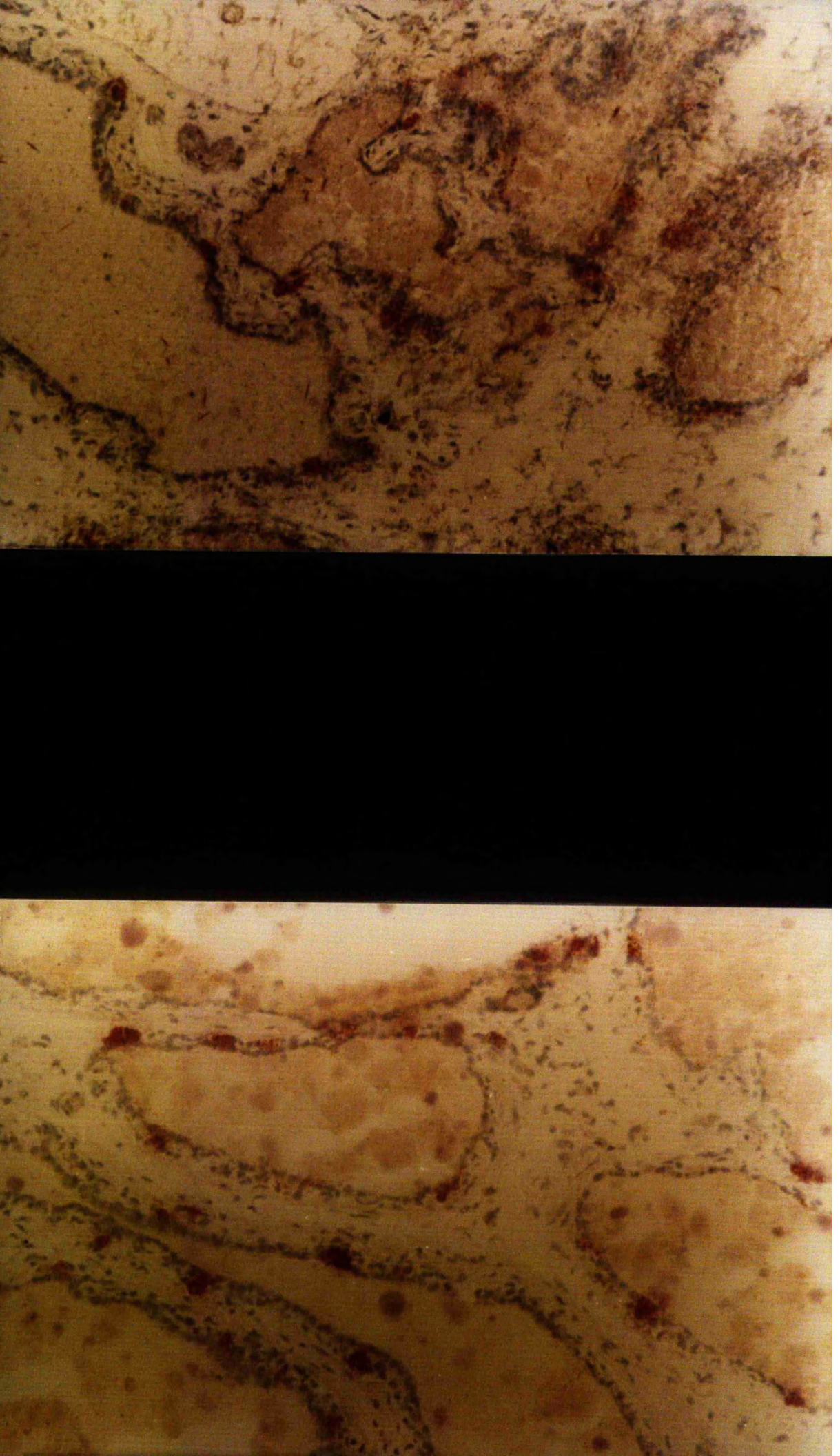


Plate 3.37 Ventral prostate from a retired breeding rat showing clusters of acid phosphatase activity in the alveolar epithelium. Acetone fixation. X160.

Plate 3.38 Section of the same tissue specimen showing a similar acid phosphatase reaction after 5h fixation in 10% neutral formalin. X160.



3.4 ISOLATION, CHARACTERIZATION AND CULTURE OF EPITHELIAL CELLS FROM RAT VENTRAL PROSTATE

Disaggregation of rat ventral prostate by enzymatic and mechanical disruption provided cell suspensions with ≥ 66% viability, as indicated by the exclusion of trypan blue dye. Microscopic examination of the cell suspension following the 60 minute incubation period indicated that the tissue was almost completely digested, with only a few small clusters of undissociated epithelial cells present. Passage of the cell suspension through a 20μm nylon sieve left no residue behind. Extension of the digestion period to 90 minutes did not further disrupt the undissociated fragments but appeared to have a damaging effect on the cell membranes as cytoplasmic protrusions were observed on the external surfaces of many cells.

Centrifugation of the cell suspension through a discontinuous Percoll density gradient (1.02 - 1.10g ml^{-1}) yielded a reproducible pattern of three visible fractions (Fig. 3.28). Fraction 1 sedimented slightly into the 1.02g ml^{-1} layer, had a flocculant appearance and consisted predominantly of cell debris (Plate 3.39). Fraction 2 appeared as a dense band at the 1.04g ml^{-1} interface and consisted primarily of large, round nucleated cells, some of which retained the characteristic appearance of prostatic epithelial cells (Plate 3.40). Fraction 3 formed a broad band which occurred between the 1.06 and 1.08g ml^{-1} layers and contained many erythrocytes along with some small and large, round nucleated cells (Plate 3.41).

On the basis of cell morphology, it appeared that

Fraction 2 was the epithelial cell enriched band. The histochemical demonstration of acid phosphatase activity in cells derived from Fraction 2 was further indicative of their epithelial origin (Plate 3.42). The intensity of the acid phosphatase staining reaction remained the same whether the cytopreparations were fixed in cold acetone or 10% neutral formalin (1 minute) or were air-dried without fixation. Epithelial cell viability was $\geq 73\%$, as indicated by the exclusion of trypan blue, and between 3.4 to 4.25 x 10^6 viable epithelial cells were isolated from each cell suspension.

A preliminary investigation of the growth potential of isolated prostatic epithelial cells in vitro showed that within one week cells derived from Fraction 2 generated numerous colonies of cells which retained histochemically demonstrable acid phosphatase activity. Coverslip cultures of isolated prostatic epithelial cells labelled with ^{3}H-TdR showed that labelling was absent during the first day of the culture period but progressively increased with time in culture (Plate 3.43). Furthermore, replicate cultures indicated that positive acid phosphatase activity was retained for at least nine days in culture (Plate 3.44). In contrast, suspension cultures of isolated prostatic epithelial cells showed that maximum ^{125}I-UdR uptake occurred during the first three days of the culture period and rapidly declined thereafter (Fig. 3.29).

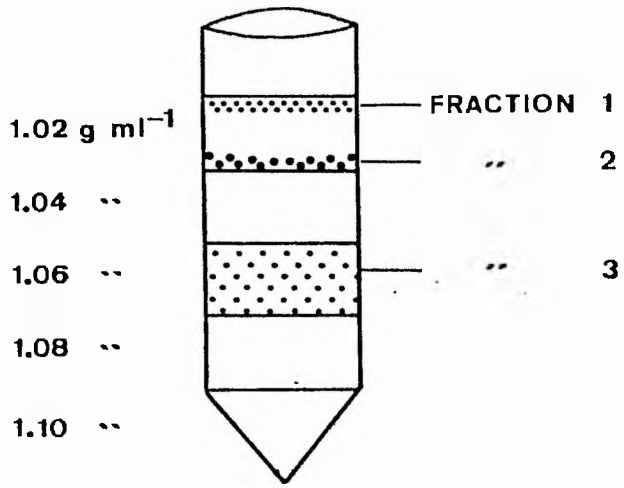


Fig. 3.28 Distribution of cells from enzymatically dissociated rat ventral prostate on the discontinuous Percoll density gradient.

Fig. 3.29 Incorporation of ^{125}I-UdR by cells derived from Fraction 2 and maintained in suspension cultures (microtest plates) for up to nine days in RPMI 1640 medium supplemented with 10% calf serum, insulin ($3\mu g\ ml^{-1}$) and 4×10^{-7}M testosterone.

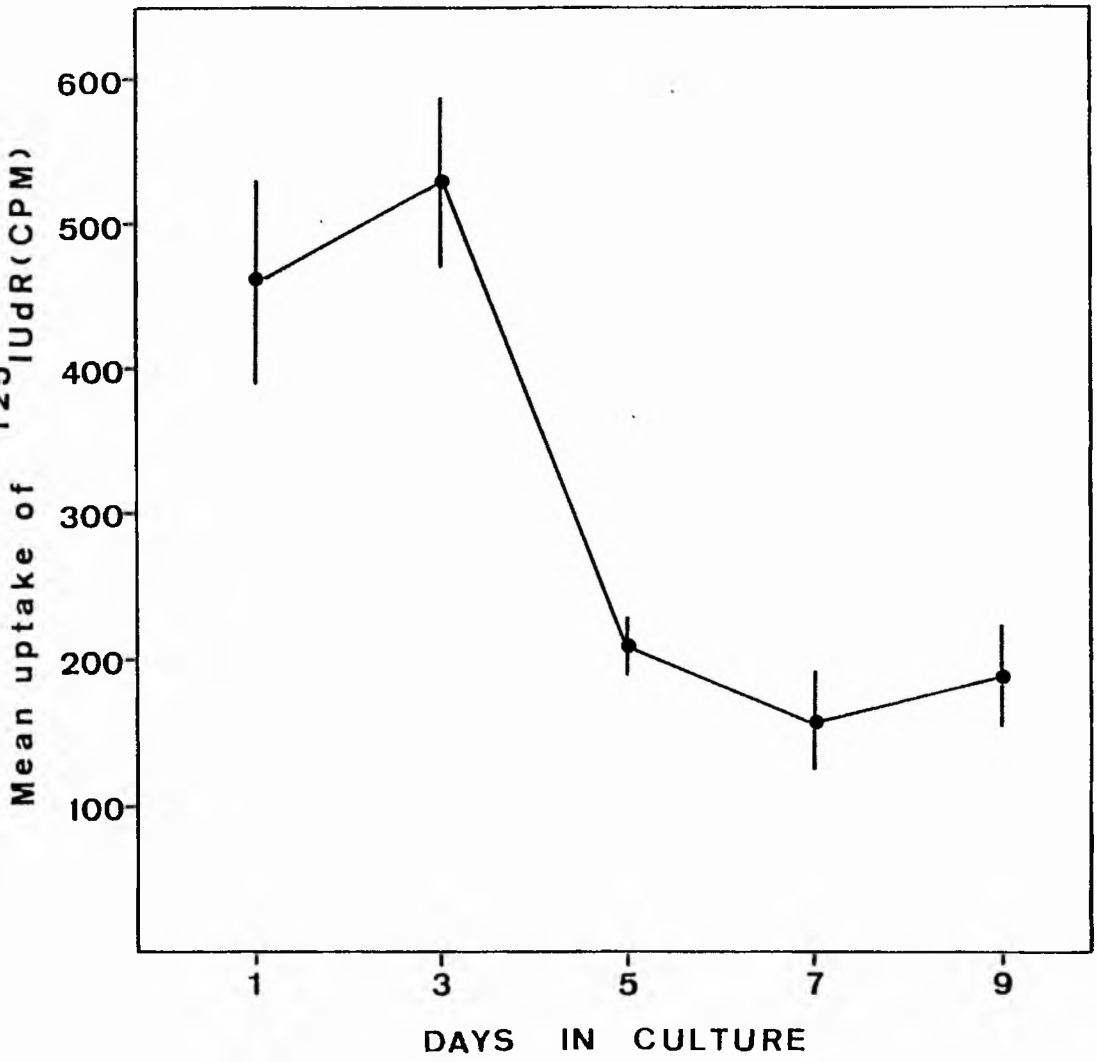

Mean uptake of ^{125}IUdR(CPM)
600
500
400
300
200
100
1
3
5
7
9
DAYS IN CULTURE

Plate 3.39 Microscopic appearance of a cytocentrifuge preparation of Fraction 1 showing primarily cellular debris. Jenner-Giemsa. X200.

Plate 3.40 Microscopic appearance of a cytocentrifuge preparation of Fraction 2 showing large, round nucleated cells, one of which (arrow) demonstrates the typical columnar shape of a prostatic epithelial cell with a negatively stained supranuclear zone. Jenner-Giemsa. X400.

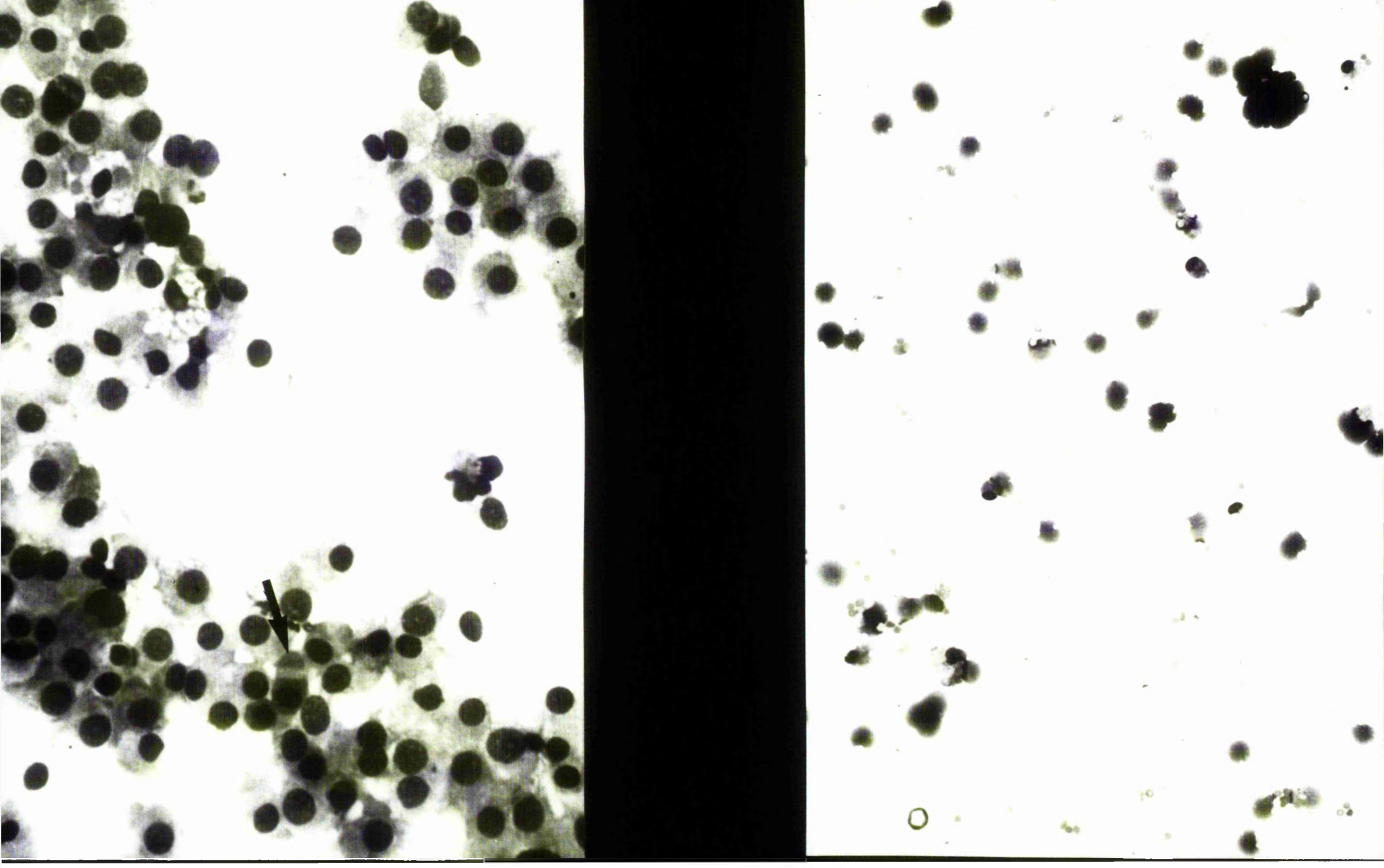
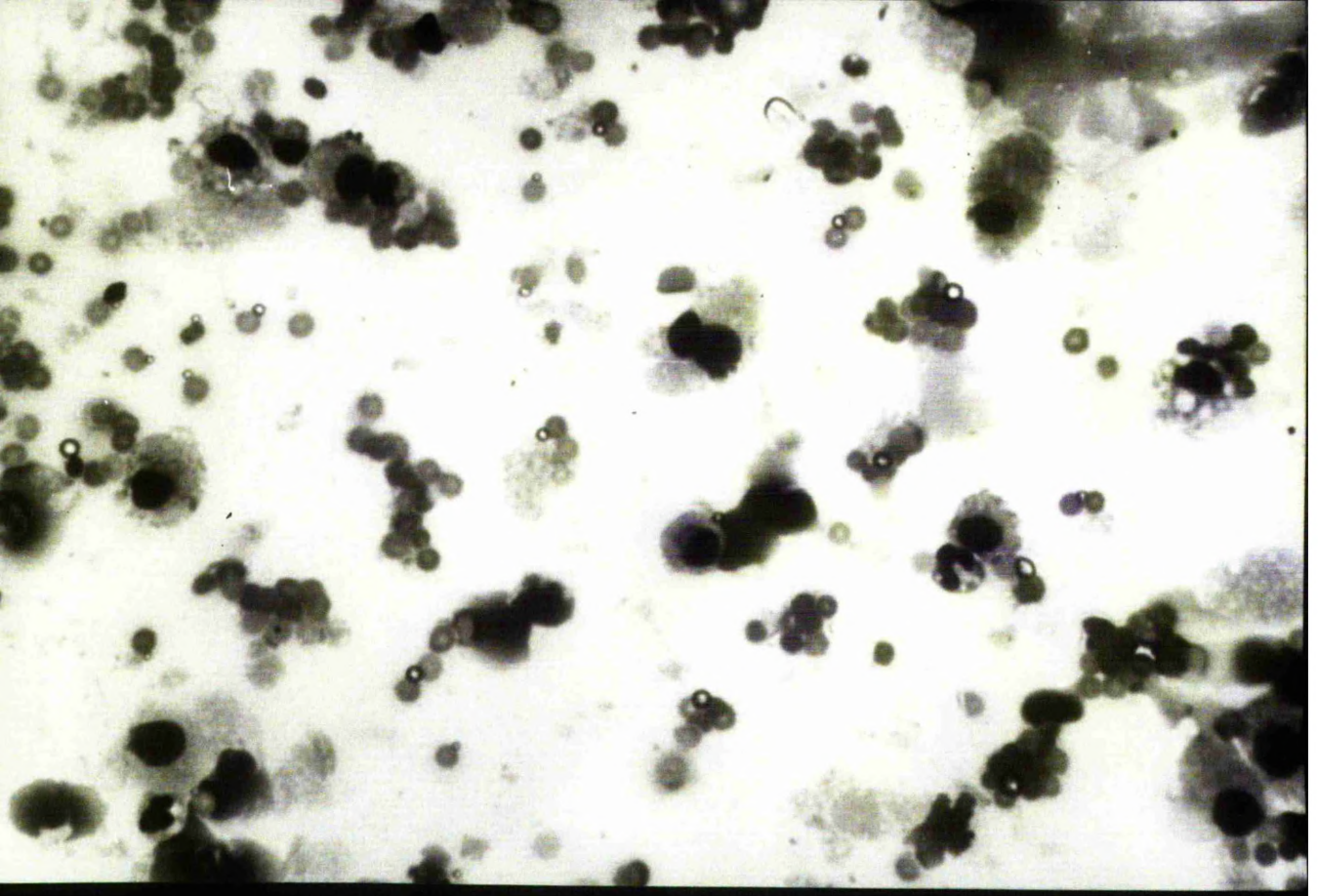


Plate 3.41 Microscopic appearance of a cytocentrifuge preparation of Fraction 3 showing many red blood cells and some large, round nucleated cells. Jenner-Giemsa. X400.

Plate 3.42 Photomicrograph of a cytocentrifuge preparation of cells derived from Fraction 2 showing positive acid phosphatase activity. Napthol AS-MX phosphate and Fast Garnet GBC diazonium salt. X160.



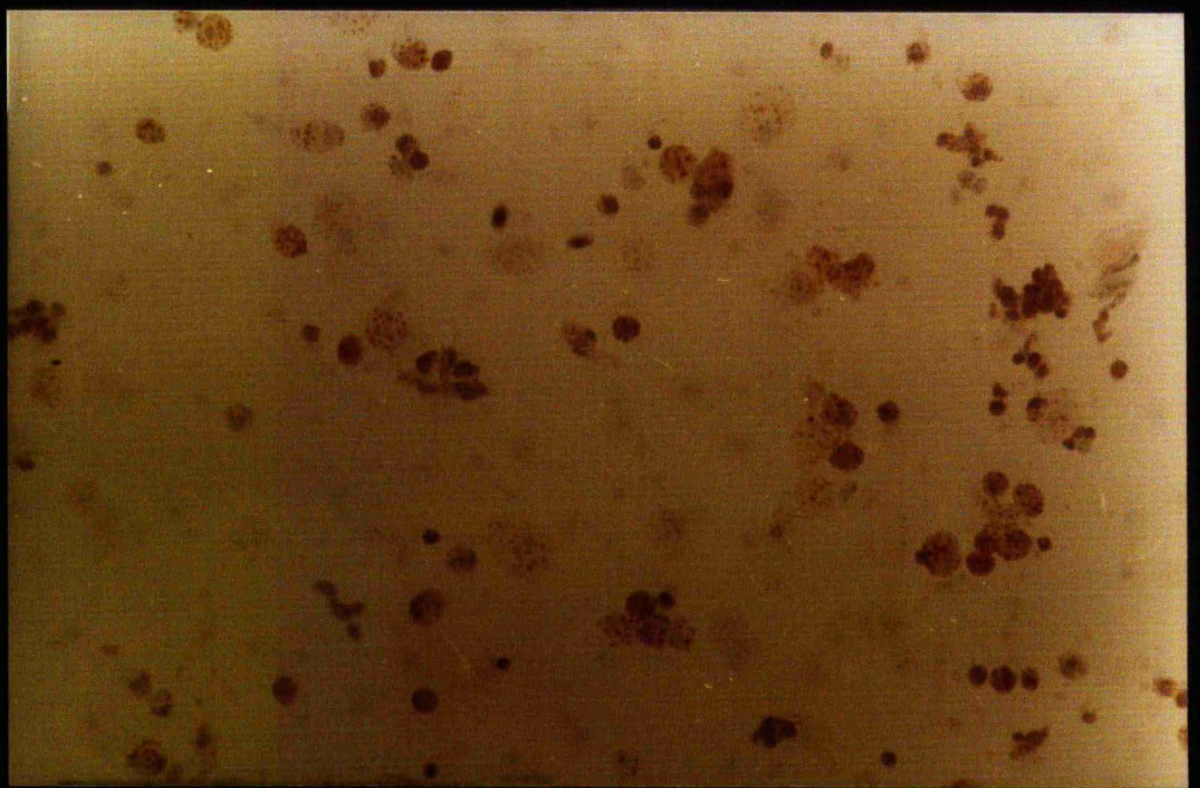
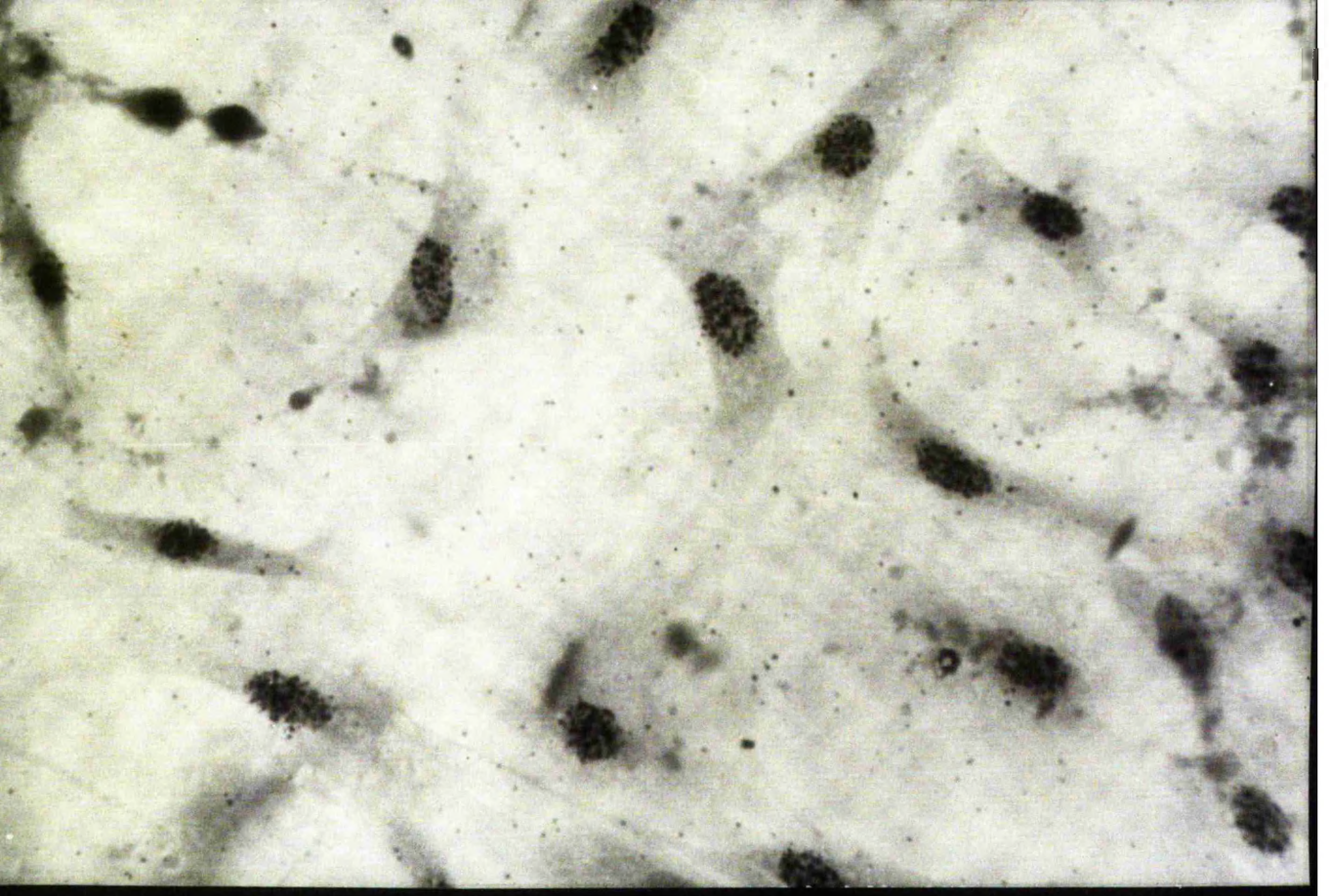
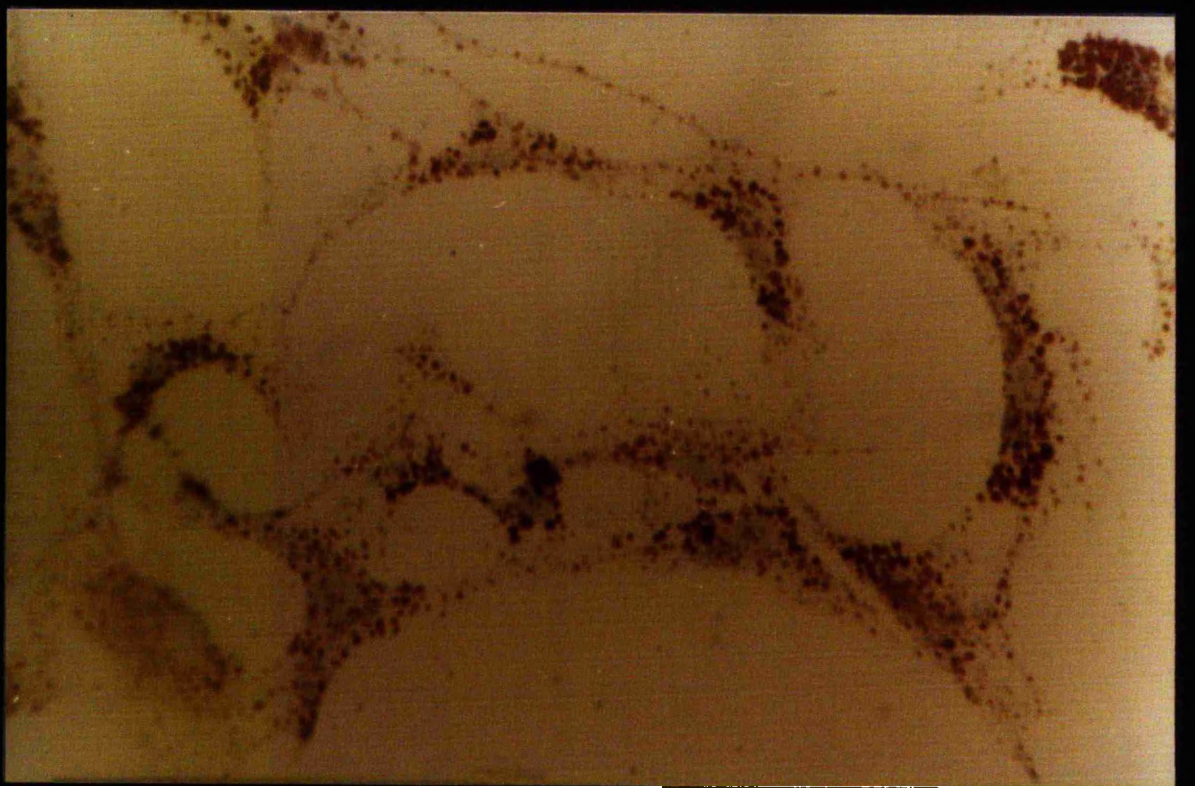


Plate 3.43 Autoradiograph of a primary monolayer culture generated by cells derived from Fraction 2 and labelled with ^{3}H-TdR after four days of culture. Haematoxylin & Eosin. X400.

Plate 3.44 Photomicrograph of a primary monolayer culture generated by cells derived from Fraction 2 and stained for acid phosphatase after four days of culture. Napthol AS-MX phosphate and Fast Garnet GBC diazonium salt. X160.





CHAPTER 4

DISCUSSION

In the present study, incorporation of the thymidine analogue, ^{125}I-UdR, has been used to monitor the proliferative activity of normal rat ventral prostate *in vitro* with the aim of establishing a model system to study the effects of hormones and chemotherapeutic agents on prostatic growth. Riches *et al.* (1976a) demonstrated the use of ^{125}I-UdR labelling as a quantitative means of monitoring DNA synthesis in organ cultures of prostatic tissue, without histoquantitation or biochemical extraction procedures. Results of the present study have further shown that good correlation exists between the ^{125}I-UdR activity monitored in whole explants of cultured rat ventral prostate and DNA biochemically extracted from replicate explants, thus confirming that the isotope is preferentially incorporated into DNA and that the activity monitored in whole explants reflects the ^{125}I-UdR uptake associated with the nuclear fraction.

As the ^{125}I-UdR labelling technique permits correlation of radioactive uptake with histological analysis of the tissue sample, the initial objective of the present study was to characterize the proliferative activity of cultured rat ventral prostate both quantitatively and qualitatively. The results have shown that in androgen-free, chemically-defined organ culture, the proliferative activity of young adult rat ventral prostate gradually declines with time in culture. Similar results have also been shown for rat prostate maintained in chemically-defined (Johansson, 1975; Donaldson and Thomas, 1976) and semi-defined (ie. serum-supplemented) (Shipman *et al.*, 1975; Riches *et al.*, 1976b) androgen-free media. However, Simnett and Morley (1967) reported that mitotic activity in the mouse (6, 14, 24 and 44 weeks old)

coagulating gland increased significantly following four days of organ culture in chemically-defined T8 medium and suggested that this response may be due to the isolation of the tissue from growth-inhibiting factors present *in vivo*. However, Müntzing and colleagues (Müntzing and Murphy, 1977; Müntzing *et al.*, 1979; Müntzing, 1980) contend that there is little experimental evidence to confirm the existence of a prostatic chalone in rat ventral prostate. Implantation of atrophied ventral prostatic tissue from inbred, orchiectomized rats into the ventral prostate of normal, inbred rats caused the DNA content of the gland to increase considerably above normal values. This suggests that there is no soluble growth inhibitor diffused in the normal prostate in a sufficient amount to prevent epithelial cell proliferation (Müntzing *et al.*, 1979). Since rat ventral prostate does not proliferate in androgen-free culture medium, it seems unlikely that a mitotic inhibiting factor present *in vivo* is lost as a result of dissection trauma or during organ culture of the prostate.

Alternatively, inherent differences between the ventral prostate and the coagulating gland may be responsible for differences in the pattern of proliferative activity *in vitro* (Donaldson and Thomas, 1976). The rat coagulating gland, unlike the ventral prostate, undergoes epithelial hyperplasia in androgen-free organ culture (Lasnitzki, 1974). *In vivo* studies have also shown that while cell renewal ceases in rat ventral prostate following castration, a low level of mitotic activity is retained in the coagulating gland (Tuohimaa and Niemi, 1974; Tuohimaa, 1980). However, the coagulating gland is a more androgen-sensitive target organ than the ventral prostate and, therefore, this residual mitotic activity *in vivo* may be related to the action of adrenal androgens (Tuohimaa, 1980).

In contrast to Simnett and Morley (1967), Franks (1961, 1963) attributed increased epithelial cell proliferation in cultured mouse prostate to the presence of insulin in T8 medium. The latter contains a high concentration of insulin (50μg ml^{-1}; Trowell, 1959) and, as will be discussed later, insulin has a well-documented stimulatory effect on the proliferative activity of rodent prostate in organ culture.

Retrogressive changes resembling those of post-castration atrophy *in vivo* have been described in both mouse (Lasnitzki, 1951, 1954, 1955, 1963, 1974; Lieber and Veneziale, 1980) and rat (Lasnitzki, 1965b, 1970a, 1970b, 1974, 1975, 1976; Lasnitzki *et al.*, 1966; Lasnitzki and Robel, 1969; Gittinger and Lasnitzki, 1972; Johansson and Niemi, 1975; Feyel-Cabanes *et al.*, 1978, 1980; Lieber and Veneziale, 1980; Sandberg and Kadohama, 1980) prostate maintained in androgen-free organ culture. The tall, folded columnar epithelium of the intact rat ventral prostate decreases in height, the supranuclear clear zone disappears, secretory activity is diminished or absent and the content of fibromuscular stroma increases (Lasnitzki, 1974). Similar changes in the epithelium of rat ventral prostate have been shown in the present study and differences between the epithelial height of intact and cultured prostatic tissue clearly demonstrate the retrogressive effect of androgen deprivation. The histological response of the tissue to androgen withdrawal also correlates well with the progressive decrease observed in ^{125}I-UdR uptake.

However, in contrast to the previously mentioned *in vivo* and *in vitro* studies of the effects of androgen deprivation on rat prostate, the present results did not show a marked increase in the content of fibromuscular stroma. Likewise, Riches *et al.* (1976b) were unable to demonstrate a significant increase in the proportion of interacinar tissue in rat prostate

cultured for four days in androgen-free, semi-defined medium. Lasnitzki (1964, 1974) reported that stromal growth in rat ventral prostate maintained in androgen-free, semi-defined medium is much less pronounced than that in completely natural medium. This suggests that the latter may contain growth promoting factors which specifically affect the stromal element. Webber (1974, 1978) has also shown that the use of horse serum in cultures of normal, sexually-immature human prostate favoured epithelial cell proliferation, whereas foetal bovine serum stimulated growth of fibroblasts, thus suggesting that the type of serum used may selectively enhance proliferation of a particular cell type. Franks (1959b, 1961, 1963) also showed that the transfer of hyperplastic explants of mouse prostate from serum-free medium to culture medium containing 20 to 40% human adult male serum causes selective degeneration of the hyperplastic epithelial cells. This further suggests that serum supplements may contain growth regulatory factors which are cell-specific.

However, marked increases in stromal growth have also been reported in cultures of rat ventral prostate maintained in chemically-defined medium for six days (Feyel-Cabanes et al., 1978, 1980). Possibly, the amount of stromal tissue observed in explants of rodent prostate is related to the duration of the culture period. Johansson and Niemi (1975) reported that rat ventral prostate cultured for two days in semi-defined medium closely resembled the in vivo control, whereas a marked increase in the content of fibromuscular stroma was evident after six days of culture. Furthermore, the present study has shown that ^{3}H-TdR labelling in four day cultures of rat ventral prostate maintained in chemically-defined medium was restricted to a few isolated fibroblasts and some small outgrowths of

peripherally located epithelium, while Feyel-Cabanes et al. (1980) found that in six day cultures of rat ventral prostate maintained in chemically-defined medium ^{3}H-TdR labelling was heavily concentrated over perialveolar and interstitial fibroblasts.

However, Lasnitzki and Robel (1969) have also emphasized that the retrogressive changes observed in cultured rat ventral prostate often vary between groups of animals. In some glands, epithelial atrophy is accompanied by increased stromal growth, whereas in others the effect is confined to regression of the alveolar epithelium. Thus, differences in the content of fibromuscular stroma may not necessarily be directly related to variations in the experimental conditions.

The addition of testosterone to organ culture media has a well-defined effect in preventing epithelial retrogression in rodent prostate (Lasnitzki, 1954, 1955, 1963, 1965b, 1970a, 1970b, 1974, 1975, 1976; Lieber and Veneziale, 1980; Sandberg and Kadohama, 1980). Testosterone maintains the height and secretory activity of the epithelium, suppresses stromal growth and stimulates epithelial cell proliferation (Lasnitzki, 1974). However, the proliferative response of rat prostate to testosterone stimulation in organ culture has most frequently been assessed on the basis of histological criteria, as few studies have attempted to quantitate the response. Lasnitzki (1970b) demonstrated that 35×10^{-6}M testosterone stimulated a marked increase in the ^{3}H-TdR labelling index and grain count of rat prostate cultured in semi-defined medium. Johansson and Santti (1973) reported that testosterone activated the incorporation of labelled DNA precursors in rat ventral prostate cultured for two to four days in chemically-defined medium. Johansson and Niemi (1975) showed significant increases in the incorporation of labelled thymidine

(^{3}H-TdR and ^{14}C-TdR) and the ^{3}H-TdR labelling index of rat ventral prostate following eight days of organ culture in semi-defined medium supplemented with 1μg ml^{-1} (3.5 x 10^{-6}M) testosterone. In contrast, Lasnitzki (1979) demonstrated that 3μg ml^{-1} (1.04 x 10^{-5}M) testosterone did not stimulate ^{3}H-TdR uptake in rat prostate cultured for four to six days in semi-defined medium. However, Johansson (1975) showed that testosterone concentrations ranging from 10^{-9} to 10^{-6}M in chemically-defined medium stimulated significant increases in ^{3}H-TdR incorporation in four day cultures of rat ventral prostate, but 10^{-5}M was inhibitory. Similarly, Johansson and Niemi (1975) showed that 0.003μg ml^{-1} (1.04 x 10^{-8}M) testosterone had a marked stimulatory effect on the incorporation of ^{3}H-TdR by cultures of rat ventral prostate maintained in chemically-defined medium for four days. Donaldson and Thomas (1976) have also shown marked stimulation of ^{125}I-UdR uptake on day 4 in cultures of rat ventral prostate maintained in the presence of insulin (3μg ml^{-1}) and testosterone (10^{-7}M) in serum-free medium. In contrast, Høisaeter (1975b) was unable to demonstrate any stimulation in the incorporation of ^{3}H-TdR by rat ventral prostate following three days of organ culture in semi-defined medium containing 4 x 10^{-6}M testosterone.

Results of the present study are in good agreement with those of Johansson (1975) and indicate that the proliferative response of rat ventral prostate to testosterone stimulation in chemically-defined medium is dose-dependent, with testosterone concentrations ranging from 4 x 10^{-9} to 4 x 10^{-6}M inducing maximal ^{125}I-UdR uptake, while higher concentrations ($\geq$ 2.5 x 10^{-5}M) exert an inhibitory effect. The pattern of the proliferative response following stimulation with an optimal concentration of testosterone (4 x 10^{-7}M) shows that a lag

phase of approximately 48 hours precedes the onset of DNA synthesis, which reaches peak activity on day 4 of the culture period and declines thereafter, despite the continued presence of testosterone. Stimulation with 10^{-8}M testosterone has been shown to elicit a comparable pattern of ^{3}H-TdR uptake in cultures of rat ventral prostate maintained in chemically-defined medium (Johansson, 1975). A similar pattern of proliferative activity has also been observed in the accessory sex glands of castrated rodents treated with exogenous testosterone (Coffey et al., 1968; Carter et al., 1972; Lesser and Bruchovsky, 1973; Sufrin and Coffey, 1973; Coffey, 1974; Tuohimaa and Niemi, 1974; Bruchovsky et al., 1975; Bruchovsky and Lesser, 1976; Alison et al., 1976; Tuohimaa, 1980; Alison and Wright, 1979a, 1981). The similarity between the proliferative response to testosterone in vivo and in chemically-defined organ culture indicates that testosterone alone is capable of promoting cell proliferation in the prostate, suggesting that other hormonal or non-hormonal factors present in vivo may serve only to modify the testosterone response. The rapid decline observed in the proliferative activity of cultured rat ventral prostate following the transitory surge of testosterone-induced DNA synthesis on day 4 is also remarkably similar to the in vivo response, which strongly suggests the presence of a specific mitotic inhibitory system. While the present study does not permit any conclusion regarding the nature of the growth-limiting mechanism, the results do indicate that prostatic growth is regulated by locally active factors which remain functional in organ culture. Furthermore, since the suppression of proliferative activity both in vivo and in vitro occurs despite the continued administration or presence of testosterone, it appears that the initial stages of the proliferative response are androgen-dependent, whereas the

latter stages of the response are independent of androgenic stimulation.

In vivo studies have also shown that in castrated rat ventral prostate, the surge of proliferative activity usually observed three to four days after the onset of testosterone treatment can be suppressed by the simultaneous administration of either protein synthesis inhibitors, such as cycloheximide or puromycin, or the antiandrogen, cyproterone acetate, with testosterone during the 48 hour latent period preceding DNA synthesis. However, delaying the onset of these inhibitors beyond this time is completely ineffective in blocking DNA synthesis, thus suggesting that androgen-mediated events necessary for the induction of the proliferative response occur during the latent period (Carter et al., 1972; Sufrin and Coffey, 1973; Coffey, 1974; Mainwaring, 1977). Similarly, the present study has shown that the addition of testosterone (4×10^{-7}M) within the first 48 hours of the culture period elicits maximal proliferative activity on day 4, whereas the addition of testosterone after this time diminishes the magnitude of the response. Delaying the onset of testosterone does not, however, shift the day of peak activity but, rather, shortens the duration of the latent period. Results of the present study have also shown that the addition of testosterone only during the first 24 hours, the second 24 hours or the first 48 hours evokes an increase in DNA synthesis on day 4 which is of equivalent magnitude to the response elicited when testosterone is present for the entire culture period. Similarly, Lesser and Bruchovsky (1973) demonstrated that in vivo a single androgen injection is capable of stimulating a marked increase in DNA synthesis in castrated rat ventral prostate. These results suggest that either androgens are very long lived in

prostatic tissue (Lesser and Bruchovsky, 1973; Bruchovsky *et al.*, 1975) or, more likely, that proliferation, once initiated, can proceed without further hormonal stimulation (Coffey, 1974).

In contrast to the present study, *in vivo* studies have shown that the magnitude of the proliferative response to androgen stimulation in the accessory sex glands of castrated rodents increases with time after castration (Österberg and Tuohimaa, 1975; Alison and Wright, 1979a, 1981; Tuohimaa, 1980) and it has been suggested that this increased proliferative activity is a reflection of the cell production required to restore pre-castration cellularity of the gland (Alison and Wright, 1979a, 1981). However, *in vivo* studies have also shown that the response to androgen treatment in the accessory sex glands of castrated rodents becomes more rapid as the time interval between castration and the onset of androgen administration increases (Saunders, 1963; Österberg and Tuohimaa, 1975; Bruchovsky and Lesser, 1976; Alison *et al.*, 1976; Alison and Wright, 1979a, 1981; Tuohimaa, 1980). Alison and Wright (1981) have suggested that as the period of androgen deprivation increases in castrated animals, cells of the accessory sex glands may move at a "shallower level within G_0", resulting in a more rapid induction of DNA synthesis and, hence, shortening of the pre-replicative phase, once the androgenic stimulus is introduced.

The dose-dependent effect of testosterone on ^{125}I-UdR uptake in cultured rat ventral prostate correlated well with the histological response of the tissue. Treatment with 4×10^{-12} to 4×10^{-10}M testosterone in chemically-defined medium was insufficient to completely prevent the occurrence of epithelial atrophy associated with androgen deprivation.

Similarly, Feyel-Cabanes et al. (1978) reported that testosterone concentrations $\geq 10^{-9}$M are required in chemically-defined medium to maintain rat ventral prostate in a state comparable to the normal, intact gland. The present results have further shown that testosterone concentrations ranging from 4×10^{-9} to 4×10^{-6}M maintained the secretory epithelium and promoted epithelial cell proliferation, particularly at the higher concentrations. Lasnitzki and Franklin (1972) and Kadohama et al. (1977) also reported that in chemically-defined organ cultures of rat prostate, testosterone concentrations ranging from 5×10^{-9} to 5×10^{-6}M are capable of providing good maintenance and stimulating epithelial cell proliferation. In the present study, the proliferative effect of testosterone (4×10^{-7}M), and the extent of the response, was further illustrated by the homogeneous distribution of ^{3}H-TdR labelling, which was associated primarily with the basal regions of the glandular epithelium. Høisaeter (1975b) has shown a similar distribution of ^{3}H-TdR labelled epithelial cells in the basal regions of alveoli in explants of rat ventral prostate cultured for four days in the presence of 4×10^{-6}M testosterone.

Results of the present study have also shown that treatment with high concentrations of testosterone (4×10^{-5}M) in chemically-defined medium has a marked cytotoxic effect on rat ventral prostate, which is presumably responsible for the dramatic decrease observed in ^{125}I-UdR uptake. Similarly, Kadohama et al. (1977) found that treatment with 5×10^{-5}M testosterone had a severe deleterious effect on rat ventral prostate cultured in chemically-defined medium. Lasnitzki (1965a) has suggested that non-specific cytotoxicity associated with the use of high hormone concentrations in vitro may be related to the concentration of the hormone per se, rather than to its

physiological function.

Apart from the concentration of testosterone, variations in organ culture media and methodology can also influence the proliferative activity of prostatic tissue *in vitro*. Merchant (1979) has emphasised the lack of uniformity in prostate culture media, particularly with respect to serum supplementation. In organ culture studies of rodent prostate, foetal calf, calf, horse or human serum are frequently incorporated into culture media, generally in concentrations ranging from 5 to 20%. However, serum supplements often have the disadvantage of being the source of pathological contaminants and of introducing biologically active substances, such as hormones, into culture media. Furthermore, the identification and concentration of such compounds is unknown and can vary greatly between batches of serum (Taylor, 1974; Sato, 1975; Hodges, 1976; Stiles *et al.*, 1981). Clearly, the introduction of unknown types and quantities of hormones through serum supplementation is of particular significance in studies attempting to define the hormone-dependence of organs, such as the prostate. In addition, most sera also contain steroid hormone binding proteins, notably TEBG, which may influence the biological action of testosterone *in vitro* by limiting the amount of free or unbound hormone available to the tissue (Lasnitzki and Franklin, 1972; Sandberg and Kadohama, 1980). Undoubtedly, the biological effects of serum will be influenced by the age, sex, species and physiological state of the donor, however, as most commercially obtained sera are pooled some of these factors are unknown or ill-defined.

In the present study, the addition of 5% foetal calf serum to the culture medium did not alter the proliferative response of rat ventral prostate to testosterone (4×10^{-7}M) stimulation or the response of testosterone-free cultures, as compared with

the serum-free system. However, in contrast, Høisaeter (1975b) was unable to demonstrate testosterone (4×10^{-6}M) stimulation of ^{3}H-TdR uptake in rat ventral prostate cultured in medium containing 5% foetal calf serum and suggested that endogenous androgens present in the serum supplement may have been responsible for masking the stimulatory effect of testosterone. These contrasting results may, however, be attributed to variations in the hormone content of different batches of commercially prepared foetal calf serum (Esber et al., 1973).

Unlike organ culture studies which have used high concentrations of testosterone ($\geq 10^{-5}$M) in semi-defined media and shown good maintenance of rat prostate (Lasnitzki, 1965b, 1970a, 1970b, 1974, 1975, 1976; Lasnitzki et al., 1966; Baulieu et al., 1968b; Lasnitzki and Robel, 1969; Robel et al., 1971), the present results have shown that treatment with 4×10^{-5}M testosterone in the presence of 5% foetal calf serum remains cytotoxic, although the severity of the toxic effect is marginally reduced compared to the serum-free system. In medium containing 10% calf serum, Johansson and Niemi (1975) found that testosterone concentrations $\geq 100\mu g\ ml^{-1}$ (3.5×10^{-4}M) also produced a non-specific cytotoxic effect in cultures of rat prostate. Differences between the effects of high concentrations of testosterone on rat prostate in organ culture may, therefore, be related to the serum supplement and, in particular, the steroid hormone binding capacity of the serum used. Lasnitzki and Franklin (1972) demonstrated that the uptake of ^{3}H-testosterone by rat ventral prostate in organ culture is inversely related to the concentration of serum used and is significantly lower in the presence of human pregnancy serum, which contains high levels of TEBG. Furthermore, Lasnitzki et al. (1974) showed that in medium containing 5% horse

serum, testosterone concentrations $\geq 3.5 \times 10^{-6}$M are required to produce an intracellular concentration of testosterone in rat ventral prostate which equals or exceeds that of the medium, whereas in serum-free medium 1.9×10^{-9}M testosterone is sufficient. Hence, supraphysiological concentrations of testosterone are routinely used in serum supplemented media in an attempt to overcome the effect of hormone binding proteins. Yet, physiological concentrations of testosterone (rat plasma testosterone approximates 10^{-8}M; Johansson, 1975) have been shown to effectively maintain rat ventral prostate cultured in either semi-defined (Johansson and Niemi, 1975) or chemically-defined (Lasnitzki and Franklin, 1972; Feyel-Cabanes *et al.*, 1978, 1980) medium. The present results have also shown that physiological concentrations of testosterone are sufficient to maintain rat ventral prostate in short-term, chemically-defined organ culture. Thus it appears that serum is not essential for the *in vitro* culture of rat prostate and its elimination from culture media may well allow more precise definition of the effects of testosterone.

Variations in the proliferative activity of rodent prostate in organ culture have also been attributed to the presence of insulin in some culture media (Franks, 1961, 1963; Donaldson and Thomas, 1976). Although insulin has been implicated as an essential factor for the culture of rodent prostate (Franks, 1961; Lostroh, 1971), it also has a well-defined stimulatory effect on the proliferative activity of rat ventral prostate *in vitro* (Johansson and Santti, 1973; Johansson, 1975; Donaldson and Thomas, 1976) and has been reported to act synergistically with testosterone in rodent prostate organ culture (Lostroh, 1968, 1971; Santti and Johansson, 1973; Johansson and Santti, 1973; Ichihara *et al.*, 1973; Fuller *et al.*, 1974; Johansson,

1975, 1976; Edwards et al., 1976; Ichihara, 1977).

Results of the present study have also shown that supplementation of chemically-defined medium with insulin ($3\mu g\ ml^{-1}$) stimulates an increase in the proliferative activity of testosterone-free cultures of rat ventral prostate. The histological response of these cultures indicated that the proliferative activity occurred in the peripherally-located alveolar epithelium, thus suggesting that insulin may promote post-traumatic regenerative hyperplasia. In contrast, Franks (1961) reported that the majority of alveoli in cultures of mouse prostate maintained in T8 medium showed evidence of epithelial hyperplasia. However, the stimulatory effect of insulin on the proliferative activity of rat ventral prostate in chemically-defined organ culture is dose-dependent (Johansson, 1975; Donaldson and Thomas, 1976) and since T8 medium contains a high insulin content, these contrasting results may be related to differences in the concentration of insulin present in the medium.

Similar to previous studies (Johansson and Santti, 1973; Johansson, 1975; Donaldson and Thomas, 1976), the present results have also demonstrated that the presence of insulin in the culture medium augments the proliferative effect of testosterone (4×10^{-7}M) on rat ventral prostate. However, treatment with 4×10^{-5}M testosterone remains inhibitory in insulin-supplemented medium. Similarly, Fuller et al., (1974) reported that insulin (0.3 and $3.0\mu g\ ml^{-1}$) enhances the stimulatory effect of 10^{-7} and 10^{-8}M testosterone on the incorporation of labelled leucine (^{14}C- or ^{3}H-leucine) and ^{3}H-uridine into cultured rat prostate, whereas 10^{-5}M testosterone has an inhibitory effect. However, the histological results of the present study have shown that treatment with

4×10^{-5}M testosterone, either in the presence or absence of insulin, is cytotoxic and presumably this accounts for reductions observed in the uptake of labelled DNA, RNA and protein precursors. Moreover, these results emphasize the importance of correlating biochemical parameters with the histological response of the tissue.

The present results have further shown that the combination of insulin with 5% foetal calf serum stimulates proportional increases in the proliferative response of rat ventral prostate, regardless of the presence (4×10^{-7} or 4×10^{-5}M) or absence of testosterone. Histologically, the results also indicated that this combination of insulin and serum provides good maintenance and stimulates epithelial cell proliferation in cultured rat ventral prostate, irrespective of whether or not testosterone is present. This suggests that insulin and serum may have a synergistic effect on rat prostate which is independent of testosterone, although it is possible that this effect is due to the synergistic action of insulin with endogenous androgens present in the serum supplement (Sandberg and Kadohama, 1980). It is, however, difficult to rationalize the stimulatory effect of insulin and serum on cultures treated with testosterone concentrations which are known to be cytotoxic in chemically-defined organ culture (ie. 4×10^{-5}M) in terms of an enhanced testosterone response.

Although insulin is known to exert a wide spectrum of effects on many cell types, the mechanism of action of this hormone remains unknown (Kahn et al., 1981). With regard to the prostate, Sandberg and Kadohama (1980) contend that insulin, itself, has no effect in the absence of androgens and suggest that, in organ culture, insulin enhances the uptake or effects

of endogenous androgens present in the explants or serum supplement. It is, however, questionable whether the explants contain sufficient levels of endogenous androgens to account for the increased proliferative activity observed in insulin-supplemented, chemically-defined organ cultures of rat prostate and, in particular, for the dose-dependence of the insulin response. Moreover, Johansson and Santti (1973) reported that in chemically-defined organ cultures of rat ventral prostate, addition of the antiandrogen, cyproterone, inhibited testosterone-induced DNA, RNA and protein synthesis, but was less effective in suppressing the stimulatory effect of insulin on these parameters. This suggests that these hormones have different mechanisms of action in the prostate. Furthermore, Lasnitzki (1974) has reported that the stimulatory effect of insulin on cultured rodent prostate is not due to increased testosterone uptake in the presence of insulin and Johansson (1976) has further shown that the rate of testosterone metabolism to 5α-dihydrotestosterone in cultured rat ventral prostate is not influenced by insulin. While insulin undoubtedly enhances the stimulatory effect of testosterone on rodent prostate *in vitro*, and in diabetic, castrated rats *in vivo* (Sufrin and Prutkin, 1974; Oksanen and Tuohimaa, 1975), it also appears that insulin may have a direct prostatic effect which is independent of androgens. Therefore, with regard to organ culture studies which aim to investigate the androgen dependence of the prostate, clearly the addition of insulin and/or serum to the culture medium complicates the interpretation of the response to androgen stimulation and deprivation. On the other hand, however, organ culture also provides an ideal method of studying interactions between testosterone and other hormonal or non-hormonal factors which may influence the effect of

testosterone on the prostate *in vivo*.

In addition to the composition of culture media, the present study has also shown that variations in organ culture methodology can influence the proliferative response of prostatic tissue *in vitro*. Rat ventral prostate cultured by submersion in non-supplemented, chemically-defined medium underwent a spontaneous surge of proliferative activity which greatly exceeded that of corresponding cultures maintained at the surface of the medium and masked the stimulatory response to testosterone (4×10^{-7}M). Regardless of the presence or absence of testosterone, submerged cultures were histologically similar in that the amount of stromal tissue was reduced, while the secretory epithelium was sloughed into the lumen and replaced by irregular, hyperplastic epithelium. In androgen-free, suspension cultures of human BPH, Webber *et al.* (1974) also reported hypocellularity of the stroma and degenerative changes in the secretory epithelium, which were accompanied by increased epithelial cell proliferation. By comparison, rat ventral prostate cultured at the gas-liquid interface of non-supplemented medium underwent epithelial atrophy typical of androgen deprivation, while similar cultures supplemented with testosterone (4×10^{-7}M) were well-maintained and showed evidence of proliferative activity beneath the intact secretory epithelium. Differences between the histological response of tissue cultured at the surface of the medium and by submersion suggest that increased ^{125}I-UdR uptake in the latter is not due to increasing the surface area of tissue exposed to the isotope. Rather, it appears that the increased proliferative activity in submerged cultures may be a form of compensatory hyperplasia, which is independent of testosterone stimulation, and may result from the degenerative effect of the culture

conditions on the secretory epithelium.

Variations in the proliferative activity of rodent prostate in organ culture can also be related to the age of the tissue. Burek (1981) has emphasized the lack of a universally acceptable definition of an "old" rat and has remarked that animals ranging from sexually-mature to thirty months of age have been classified as old, depending on the nature of the investigation. Yet, striking histological changes occur in the prostate glands of mice (Franks, 1959a, 1967) and rats (Burek, 1981) during senescence. Unlike the tall, columnar epithelium typical of young, sexually-mature rodent prostate, the histological appearance of the alveolar epithelium in aged prostatic tissue is highly variable. In senescent rat ventral prostate much of the epithelium is atrophic, while other regions appear to be normal and still other regions are apparently hyperplastic (Burek, 1981). In the present study, similar age-related changes were observed in ventral prostate from retired breeding rats (> 12 months old).

The similarity between age-associated prostatic atrophy and that which occurs following castration has prompted the widespread belief that androgen depletion may play a critical role in the onset of these morphological alterations (Mainwaring and Brandes, 1974). Although age-related changes in plasma testosterone levels have been extensively studied in the human male (Mawhinney and Belis, 1976; Voigt and Krieg, 1978; Menon and Walsh, 1979; Mann and Lutwack-Mann, 1981; Gupta, 1982), changes in plasma androgens in other species have received little attention. Lloyd (1972) has, however, reported that in the rat, blood testosterone levels increase nearly three-fold from three to seven months of age, but decrease approximately two-fold from seven to thirteen months. Thus, reductions in

total plasma testosterone levels or, possibly, alterations in the testosterone:oestradiol ratio, may be responsible for the onset of atrophic changes in the senile rat prostate.

While quantitative studies have not confirmed increased proliferative activity in aged rodent prostate (Mainwaring, 1967; Simnett and Morley, 1967; Donaldson and Thomas, 1976), histological indications of hyperplastic growth in the alveolar epithelium of senescent rodent prostate (Franks, 1959a; Burek, 1981) are of particular significance since the normal, young adult gland rarely exhibits mitotic activity in vivo (Simnett and Morley, 1967; Tuohimaa and Niemi, 1968, 1974; Donaldson and Thomas, 1976). On the basis of such histological implications, Mainwaring and Brandes (1974) suggested that age-associated changes observed in the ultrastructure of the basement membrane in rodent prostate may disrupt normal physicochemical interactions between the glandular epithelium and stroma, thereby resulting in abnormal growth.

Age-associated changes in the response of rodent prostate to testosterone stimulation in organ culture have also been reported. Lasnitzki (1954, 1974) found that in cultures of young (4 to 5 weeks old) mouse ventral prostate, testosterone maintained structural differentiation and secretory activity, but caused epithelial hyperplasia in tissue from older (5 to 6 months old) animals. However, Franks (1959a) reported that in cultures of prostatic tissue from old (8 months and 2 years old) mice, epithelial growth and secretory activity were less responsive to testosterone stimulation than in tissue from younger (5 to 6 months old) animals. This suggests that during senescence the prostate becomes less sensitive to testosterone. Mainwaring and Brandes (1974) have proposed that this decreased sensitivity to testosterone may be related to disruptions in the

normal intracellular metabolism of testosterone caused by the degradation of important cytoplasmic organelles, notably ribosomes and mitochondria, with advancing age. Biochemical studies of the *in vitro* metabolism of testosterone in ventral prostatic tissue from young (3 months old) and old (> 12 months old) rats have shown that 5α-reductase activity is markedly lower in the latter (Shimazaki *et al.*, 1969; Nozu and Tamaoki, 1974a; Mawhinney and Belis, 1976) and is accompanied by a significant reduction in the formation of 5α-dihydrotestosterone (Mawhinney and Belis, 1976). Lasnitzki (1976) also found that in organ cultures of ventral prostate from old (20 months old) rats the uptake of ^{3}H-testosterone and the formation of 5α-dihydrotestosterone was less than that in younger (2 months old) tissue. A profound decrease in cytoplasmic and nuclear androgen receptor content, accompanied by a diminished capacity to synthesize 5α-dihydrotestosterone, has also been reported in aging rat ventral prostate (Shain and Axelrod, 1973; Shain *et al.*, 1977). These results suggest that the reduced sensitivity of aged rat ventral prostate to testosterone may be related to important age-related changes in the ability of the tissue to accumulate, retain and/or metabolize testosterone.

Results of the present study have also shown that the proliferative response of aged rat ventral prostate is less sensitive to testosterone stimulation at all concentrations which were effective in younger tissue. However, the results also showed that aged prostatic tissue cultured in the absence of testosterone undergoes a spontaneous surge of proliferative activity, which may mask the effect of testosterone. Androgen-free cultures of human BPH also exhibit a similar burst of proliferative activity, which may be representative of a wound

healing effect caused by dissection trauma (McMahon and Thomas, 1973; Shipman et al., 1975; Donaldson and Thomas, 1976; Riches et al., 1976b, 1982; Mistry et al., 1982). However, in organ culture studies of rodent prostate, post-traumatic regeneration is usually associated with peripherally located alveolar epithelium (Franks, 1961, 1963; Lasnitzki, 1965b), whereas in the present study, cultures of aged prostatic tissue demonstrated irregular, hyperplastic epithelium throughout the explant, both in the presence and absence of testosterone. In addition, stromal tissue in these cultures showed marked degenerative changes. Since explantation of aged prostatic tissue required greater force than younger tissue, it is possible that the older tissue was subjected to greater trauma, which may further disrupt epithelial - stromal relationships in aged rodent prostate, thereby resulting in increased epithelial cell proliferation in vitro.

While the present study does not permit any conclusions with regard to factors which may be involved in the age-related decrease in sensitivity of rodent prostate to testosterone, it does demonstrate that the proliferative activity of aged rat ventral prostate maintained in androgen-free organ culture differs markedly from that of younger tissue and that aged prostatic tissue no longer remains clearly responsive to testosterone stimulation or deprivation. Organ culture studies of human BPH have experienced similar difficulty in establishing well-defined responses to either androgen stimulation or withdrawal (McMahon and Thomas, 1973; Harbitz, 1973; McRae et al., 1973; Harbitz et al., 1974; Lasnitzki et al., 1975; Donaldson and Thomas, 1976; Riches et al., 1976b, 1982; Lasnitzki, 1979; Mistry et al., 1982). While aged rat ventral prostate may be most representative of human BPH, the similarity

between the response of aged rat prostatic tissue cultured in either the presence or absence of testosterone precludes its use as a suitable model for *in vitro* investigations of androgen-dependent responses in the prostate.

In normal young adult rat ventral prostate testosterone is subjected to extensive metabolism both *in vivo* (Bruchovsky and Wilson, 1968a, 1968b; Anderson and Liao, 1968) and *in vitro* (Baulieu *et al.*, 1968a, 1968b, 1969; Robel *et al.*, 1971, 1975). Organ culture studies have shown that testosterone and its metabolites all promote varying degrees of epithelial cell proliferation, but the principal metabolite, 5α-dihydrotestosterone, is the most potent mitogenic agent, while 5α-androstane-3β,17β-diol provides good maintenance of the secretory epithelium but never induces marked epithelial hyperplasia (Baulieu *et al.*, 1968a, 1968b, 1969; Lasnitzki, 1970a, 1970b, 1971, 1974, 1975, 1976; Robel *et al.*, 1971, 1975). Thus, Baulieu *et al.* (1968a, 1968b) suggested that the androgenic effect of testosterone in rat prostate is mediated via its intracellular metabolites and that 5α-dihydrotestosterone promotes cell proliferation while 5α-androstane-3β,17β-diol functions in the maintenance of cytodifferentiation.

Few studies have attempted a quantitative comparison of the effects of testosterone and its metabolites on the proliferative activity of rat prostate in organ culture. However, Lasnitzki (1970b) demonstrated that 17.5 x 10^{-6}M 5α-dihydrotestosterone stimulated marked increases in the ^{3}H-TdR labelling index and grain count of rat prostate cultured in semi-defined medium, whereas a higher concentration of testosterone (35 x 10^{-6}M) was required to elicit a comparable response, and 5α-androstane-3β,17β-diol was not stimulatory even at the highest concentration (70 x 10^{-6}M). Lasnitzki (1979) also

showed that 3μg ml^{-1} (1.04 x 10^{-5}M) of 5α-dihydrotestosterone stimulated an increase in the ^{3}H-TdR labelling index of rat prostate cultured for four to six days in semi-defined medium, whereas an equivalent concentration of testosterone was ineffective. In contrast, Johansson and Niemi (1975) demonstrated that 1μg ml^{-1} (3.5 x 10^{-6}M) testosterone and 5α-dihydrotestosteron had similar stimulatory effects on the incorporation of labelled thymidine (^{14}C-TdR or ^{3}H-TdR) in eight day cultures of rat ventral prostate maintained in semi-defined medium, whereas 5α-androstane-3β,17β-diol elicited a weaker stimulatory response. Johansson and Niemi (1975) also showed that in chemically-defined medium 0.003μg ml^{-1} (1.04 x 10^{-8}M) testosterone or 5α-dihydrotestosterone stimulated similar increases in ^{3}H-TdR uptake in four day cultures of rat ventral prostate while 5α-androstane-3β,17β-diol was less effective. Results of the present study have further shown that equimolar concentrations (4 x 10^{-9} or 4 x 10^{-7}M) of testosterone, 5α-dihydrotestosterone, androstenedione and androstanedione all stimulated comparable increases in the uptake of ^{125}I-UdR by cultures of young adult rat ventral prostate maintained in chemically-defined medium for four days and that at 4 x 10^{-5}M these androgens were all equally inhibitory. In contrast, 5α-androstane-3β,17β-diol was not stimulatory at 4 x 10^{-9}M and at both 4 x 10^{-7} and 4 x 10^{-5}M it induced marginal, yet comparable, increases in ^{125}I-UdR uptake.

The histological results of the present study correlated well with the incorporation of ^{125}I-UdR, indicating that at optimal concentrations testosterone, 5α-dihydrotestosterone, androstenedione, and androstanedione all preserved the secretory epithelium and promoted epithelial cell proliferation to a similar extent. Likewise, Roy et al. (1972a) found that equimolar concentrations (35 x 10^{-6}M) of testosterone,

androstenedione and androstanedione had similar effects on the induction of epithelial cell proliferation in cultures of rat ventral prostate maintained in semi-defined medium. Similarly, Johansson and Niemi (1975) reported that equivalent concentrations (0.02 to 10μg ml^{-1}; 6.9 x 10^{-8} to 3.5 x 10^{-5}M) of testosterone and 5α-dihydrotestosterone had comparable effects on the maintenance of the alveolar epithelium and stimulation of epithelial cell proliferation in semi-defined organ cultures of rat ventral prostate. These results are, however, in contrast to earlier studies (Baulieu et al., 1968a, 1968b, 1969; Lasnitzki, 1970a, 1970b, 1971, 1974, 1975, 1976; Robel et al., 1971, 1975) which indicated that 5α-dihydrotestosterone was consistently more effective than either testosterone or androstanedione in promoting epithelial hyperplasia in cultures of rat prostate.

Results of the present study have also shown that, unlike testosterone, 5α-dihydrotestosterone, androstenedione or androstanedione, treatment with 4 x 10^{-9}M 5α-androstane-3β,17β-diol did not prevent the occurrence of epithelial atrophy associated with androgen deprivation, but at both 4 x 10^{-7} and 4 x 10^{-5}M it fully maintained the height and secretory activity of the epithelium without causing marked epithelial cell proliferation. Furthermore, among all the testosterone metabolites studied, only 5α-androstane-3β,17β-diol was not toxic at 4 x 10^{-5}M. Similarly, Johansson and Niemi (1975) reported that, unlike testosterone and 5α-dihydrotestosterone, low concentrations of 5α-androstane-3β,17β-diol (0.02 to 0.1μg ml^{-1}; 6.9 x 10^{-8} to 3.5 x 10^{-7}M) were unable to prevent epithelial retrogression in semi-defined organ cultures of rat ventral prostate and that at high concentrations ($\geq$100μg ml^{-1}; 3.5 x 10^{-4}M) both testosterone and 5α-dihydrotestosterone became cytotoxic, whereas 5α-androstane-3β,17β-diol did not. However, in contrast

to previous studies (Baulieu et al., 1968a, 1968b, 1969; Robel et al., 1971; Lasnitzki, 1970a, 1970b, 1971, 1974, 1975, 1976) and the present results, Johansson and Niemi (1975) were unable to detect qualitative differences in the proliferative response of rat ventral prostate to stimulation with 10μg ml^{-1} (3.5 x 10^{-5}M) testosterone, 5α-dihydrotestosterone or 5α-androstane-3β,17β-diol in semi-defined organ culture. On the basis of this observation, Johansson and Niemi (1975) contend that these steroids are qualitatively similar, despite clear differences between the biological effects of 5α-androstane-3β,17β-diol and those of testosterone and 5α-dihydrotestosterone at both low (0.02 to 0.1μg ml^{-1}; 6.9 x 10^{-8} to 3.5 x 10^{-7}M) and high concentrations ($\geq$100μg ml^{-1}; 3.5 x 10^{-4}M). They suggest that testosterone and its metabolites activate a common biological mechanism, but with varying degrees of effectiveness. Variations in the androgenic effects of testosterone and its metabolites have been attributed to differences in their steric configuration, which may affect their binding affinity to receptors in the prostate (Saunders, 1963; Liao et al., 1973; Mainwaring, 1977; Tuohimaa, 1980). Organ culture studies have shown that 5β-dihydrotestosterone, unlike its epimer 5α-dihydrotestosterone, has no effect on the stimulation of cell proliferationor maintenance of rat prostate in vitro (Baulieu et al., 1968a, 1968b; Robel et al., 1971). Similarly, the present study has shown that 5β-dihydrotestosterone was inactive at all concentrations used, and was not cytotoxic at 4 x 10^{-5}M. These results suggest that while the effect of different androgens may well vary with their configuration, the comparison of the effects of such stereoisomers is of limited value since, as Baulieu et al. (1968a) emphasize, 5β-dihydrotestosterone is not a natural product of testosterone

metabolism in the rat prostate. In addition, direct correlations between the configuration of testosterone and its 5α-metabolites with the histological response of the prostate are of limited value in organ culture without consideration of their further metabolism within the target tissue.

In contrast to Johansson and Niemi (1975), Baulieu and colleagues (Baulieu et al., 1968a, 1968b, 1969; Robel et al., 1971, 1974, 1975) suggest that distinct biological responses of the rat prostate to testosterone are mediated by various active intracellular metabolites, whereby 5α-dihydrotestosterone specifically controls cell proliferation and 5α-androstane-3β,17β-diol maintains cytodifferentiation. The metabolism of testosterone to 5α-dihydrotestosterone in cultured rat prostate has been well documented (Baulieu et al., 1968a, 1968b; Robel et al., 1971, 1975; Lasnitzki, 1971, 1974, 1976, Johansson, 1976) and Roy et al. (1972a) have further shown that both androstanedione and androstenedione are converted to 5α-dihydrotestosterone, whereas 5α-androstane-3β,17β-diol is primarily converted to the weak androgenic steroid, epiandrosterone (Robel et al., 1971, 1975; Roy et al., 1972b). This suggests that the proliferative activity ascribed to testosterone, androstenedione and androstanedione may be related to the intracellular formation of 5α-dihydrotestosterone. The similarity between the proliferative responses of rat ventral prostate to testosterone and these metabolites, as observed in the present study, strongly support this concept and, thus, corroborate the hypothesis that 5α-dihydrotestosterone is responsible for cell proliferation in rat prostate. Lasnitzki (1974) has further suggested that the ability of 5α-dihydrotestosterone to preserve cytodifferentiation in cultured rat prostate may be related to the intracellular metabolism of 5α-dihydrotestosterone to 5α-androstane-3β,17β-diol.

However, it is not yet known if the amount of 5α-androstane-3β,17β-diol formed by the prostate is sufficient to be physiologically active (Lasnitzki, 1974) nor is its mode of action resolved (Robel et al., 1974; Voigt et al., 1975; Lasnitzki, 1976). Evidence that 5α-androstane-3β,17β-diol is capable of maintaining the cytoplasmic and secretory integrity of the rat prostate in organ culture, without causing pronounced epithelial growth or becoming cytotoxic at high concentrations does, however, suggest that it may have a specific biological function, as well as a distinct mechanism of action. However, evidence indicating that a small, but significant, amount of 5α-androstane-3β,17β-diol is converted to 5α-dihydrotestosterone in the rat prostate suggests that 5α-androstane-3β,17β-diol may act partially via 5α-dihydrotestosterone (Voigt et al., 1975; Takyi et al., 1977). This may account for the marginal increase in proliferative activity observed at high concentrations of 5α-androstane-3β,17β-diol in organ culture.

Testosterone-stimulated DNA synthesis in the accessory sex glands of castrated rodents has been widely used to investigate the antiproliferative activity of chemotherapeutic agents (Carter et al., 1972; Sufrin and Coffey, 1973, 1975; Sloan et al., 1975; Forsberg and Høisaeter, 1975; Høisaeter, 1975b, 1976b, 1977; Alison and Wright, 1979c, 1980). Using a similar approach, the present study examined the effects of antiandrogens, oestrogens and hormone-cytotoxic compounds on the proliferative response induced by testosterone in organ cultures of young adult rat ventral prostate. Results of the present study have shown that treatment with testosterone (4×10^{-9} to 4×10^{-6}M) produces maximal increases (approximately 3-fold) in ^{125}I-UdR uptake on day 4 of the culture period, hence this time-point was chosen for investigation of the drug effects.

The potent antiandrogen, cyproterone acetate, has been shown to effectively block testosterone-induced DNA synthesis in castrated rat ventral prostate *in vivo* (Carter *et al.*, 1972; Sufrin and Coffey, 1973, 1975; Coffey, 1974; Coffey and Sloan, 1975; Sloan *et al.*, 1975) and is believed to exert its antiandrogenic effect primarily by direct competitive antagonism with androgens at the receptor level of the target tissue (Fang *et al.*, 1969; Fang and Liao, 1969, 1971; Belham and Neal, 1971; Walsh and Korenman, 1971; Liao *et al.*, 1974; Neumann and Steinbeck, 1974; Mainwaring, 1977; Menon and Walsh, 1979; Mittelman, 1979; Neumann *et al.*, 1976, 1982). Investigation of the direct effect of cyproterone acetate on the proliferative response of rat ventral prostate to testosterone stimulation in chemically-defined organ culture therefore permits more precise insight into the mode of action of this compound in a system which is devoid of complicating systemic factors present *in vivo*. In the present study, the simultaneous addition of 4×10^{-9} or 4×10^{-7}M cyproterone acetate with 4×10^{-7}M testosterone was found to have no effect on the stimulatory action of testosterone, whereas treatment with 4×10^{-5}M cyproterone acetate had a pronounced inhibitory effect. The histological results paralleled the ^{125}I-UdR data, indicating that in cultures treated with concentrations of cyproterone acetate less than or equal to that of testosterone, the androgen effect predominated. In contrast, treatment with 4×10^{-5}M cyproterone acetate caused extensive epithelial and stromal necrosis, indicative of a non-specific cytotoxic response. As this response is similar to that observed following treatment with high concentrations of testosterone (4×10^{-5}M) in chemically-defined organ culture, inhibition of the testosterone-induced proliferative response by 4×10^{-5}M cyproterone acetate cannot be directly attributed to specific

antiandrogenic properties of the drug. Rather, it appears that the cytotoxic response is due to the use of high concentrations of cyproterone acetate *in vitro*. *In vivo* studies have shown that in order to demonstrate antiandrogenic effects of cyproterone acetate on testosterone-induced DNA synthesis in castrated rat ventral prostate, it is necessary to use the minimum dose of testosterone required for a maximum response and to administer at least a 30-fold excess of cyproterone acetate (Carter *et al.*, 1972; Sufrin and Coffey, 1973; Coffey, 1974). Similarly, Neumann *et al.* (1980) showed a dose-dependent decrease in the seminal vesicle weight of normal male rats treated with increasing doses of cyproterone acetate (0.3 to 10.0mg/rat) and a 10-fold excess of the antiandrogen produces almost 100% inhibition of the androgen effect. Thus, using the minimum dose of testosterone required for a maximum response on day 4 (ie. 4×10^{-9}M), the present results showed that treatment with 4×10^{-7}M cyproterone acetate inhibited the testosterone-induced proliferative response and caused marked epithelial atrophy, resembling that observed in androgen-free control cultures. Likewise, Feyel-Cabanes *et al.* (1980) reported that in chemically-defined organ cultures of young adult rat ventral prostate, treatment with 1×10^{-6}M cyproterone acetate in conjunction with 1×10^{-9} to 4×10^{-9}M testosterone markedly suppressed the androgenic effect. Since cyproterone acetate does not inhibit 5α-reductase activity in rat prostate (Belham and Neal, 1971; Walsh and Korenman, 1971; Liao *et al.*, 1974; Neumann and Steinbeck, 1974; Tveter *et al.*, 1980), these results tend to corroborate the hypothesis that cyproterone acetate acts via direct competitive antagonism with androgens at the receptor level of the prostate. In contrast, Rajalakshmi and Prasad (1976) found that long-term

treatment of rats with cyproterone acetate resulted in marked suppression of 5α-dihydrotestosterone formation in the ventral prostate, thereby suggesting that the antiandrogen inhibits 5α-reductase activity. However, as 5α-reductase activity is dependent upon androgenic stimulation, chronic cyproterone acetate treatment may reduce the enzymatic activity not by enzyme inhibition but, rather, as a result of the castration-like changes induced (Tveter et al., 1980).

Using the same procedure, the present study compared the effects of the hormone-cytotoxic agent, Estracyt (estramustine phosphate disodium and estramustine phosphate) and its metabolite, estramustine with that of their carrier-hormone, oestradiol-17β, on the proliferative response to testosterone in cultured rat ventral prostate. The results have shown that treatment with oestradiol-17β was consistently more effective than either estramustine phosphate disodium, estramustine phosphate or estramustine in suppressing the testosterone-induced response. The three estramustine compounds elicited similar responses under all experimental conditions and were only effective in reducing the proliferative response to 4×10^{-9}M testosterone at a concentration of 4×10^{-5}M. The histological results further confirmed that only cultures treated with the estramustine compounds at 4×10^{-5}M in the presence of 4×10^{-9}M testosterone, showed evidence of epithelial atrophy. Treatment with these compounds did not appreciably alter retrogressive changes observed in androgen-free control cultures. Furthermore, pre-treatment (48 hours) with the estramustine compounds did not enhance their ability to inhibit the proliferative response to 4×10^{-9}M testosterone and, their addition 48 hours after that of testosterone appeared to reduce the magnitude of the inhibitory response. In contrast, treatment with oestradiol-17β at

concentrations of 4×10^{-9}, 4×10^{-7} and, in particular, 4×10^{-5}M were capable of suppressing proliferative responses to both 4×10^{-9} and 4×10^{-7}M testosterone. While the histological results demonstrated that 4×10^{-5}M oestradiol-17β exerts a non-specific cytotoxic effect in chemically-defined organ cultures of rat ventral prostate, indicating that the inhibitory effect on the proliferative response is not directly attributable to specific antiandrogenic properties of the oestrogen, the present results have further shown that treatment with lower concentrations of oestradiol-17β (4×10^{-9} and 4×10^{-7}M) evoke retrogressive changes in cultures treated with either 4×10^{-9} or 4×10^{-7}M testosterone. Furthermore, these changes become more pronounced as the concentration of testosterone decreases, thereby suggesting that oestradiol-17β is capable of directly antagonizing testosterone action at the target tissue level. Similarly, Lasnitzki (1974, 1975) reported that in semi-defined organ cultures of young adult rat ventral prostate, treatment with oestradiol-17β (0.5 g ml^{-1}; 1.8×10^{-6}M) in a ratio of 1:10 with testosterone (5.0 g ml^{-1}; 1.7×10^{-5}M) counteracted the androgen effect, resulting in marked epithelial atrophy and loss of secretory function. These results are, however, in contrast to those of Feyel-Cabanes et al. (1978, 1980), who found that treatment with oestradiol-17β (1×10^{-9} to 1×10^{-6}M had no antagonistic effect on the action of testosterone (1×10^{-9} to 4×10^{-9}M) in chemically-defined organ cultures of young adult rat ventral prostate.

By comparison with oestradiol-17β , the present results have also shown that the non-steroidal oestrogen, diethylstilboestrol, is cytotoxic at 4×10^{-5}M, but at lower concentrations (4×10^{-7} and 4×10^{-9}M) it is completely ineffective in reducing the proliferative response to testosterone (4×10^{-7}M). Similarly,

Feyel-Cabanes et al. (1978, 1980) found that the androgen effect predominated in chemically-defined organ cultures of rat ventral prostate treated with diethylstilboestrol (0.1×10^{-9} to 1×10^{-6}M) in the presence of 4×10^{-9}M testosterone. The present results, therefore, suggest that diethylstilboestrol, unlike oestradiol-17β , is unable to exert a direct antiandrogenic effect on the prostate. Oestradiol-17β reportedly exhibits high binding affinity for androgen receptor proteins in rat ventral prostate (Liao et al., 1972; Høisaeter, 1974; Blondeau et al., 1975; Baulieu et al., 1975a), whereas diethylstilboestrol shows only negligible affinity (Baulieu et al., 1975a), thus suggesting that oestradiol-17β may inhibit androgen action in the prostate by direct competition at the receptor level. While specific oestradiol-17β receptor proteins have also been demonstrated in rat ventral prostate (Jungblut et al., 1971; Baulieu et al., 1975a; O'Toole et al., 1975; Karr and Sandberg, 1979; Jung-Testas et al., 1981), diethylstilboestrol generally exhibits high binding affinity for all known oestrogen receptors (Blondeau et al., 1975; Baulieu et al., 1975a; Feyel-Cabanes et al., 1980). Therefore it seems unlikely that the oestradiol-17β effect is mediated via the oestrogen receptor. Alternatively, oestradiol-17β is known to inhibit rat prostatic 5α-reductase activity (Shimazaki et al., 1972; Lee et al., 1973a, 1973b; Baulieu et al., 1975a; Johansson, 1976; Kadohama et al., 1977) and may, therefore, exert its antiandrogenic activity by limiting the intracellular metabolism of testosterone to 5α-dihydrotestosterone. Furthermore, in comparing the effects of several oestrogens, including oestradiol-17β and diethylstilboestrol, on the 5α-reduction of testosterone in minces of rat prostate in vitro, Lee et al. (1973a, 1973b) found that the natural oestrogen, oestradiol-17β, was a far more potent inhibitor than diethylstilboestrol and suggested that

inhibition of 5α-reductase activity in the rat prostate is dependent upon a complete steroid structure and a free phenolic hydroxyl group at carbon-3. In contrast, Groom et al. (1971) and Nozu and Tamaoki (1974b) have reported that both oestradiol-17β and diethylstilboestrol inhibit rat prostatic 5α-reductase activity equally. However, more recent studies have shown that the inhibitory effect of oestradiol-17β is highly specific in that it only inhibits 5α-reductase activity in tissue from the dorsolateral lobe of the rat prostate and has little or no effect on the rat ventral prostate (Yamanaka et al., 1975; Kadohama et al., 1977). Similarly, Belham and Neal (1971) reported that diethylstilboestrol had little effect on the metabolism of ^{3}H-testosterone by rat ventral prostate. Although these results tend to suggest that the inhibitory effect of oestradiol-17β observed in the present study is not related to inhibition of 5α-reductase activity, direct comparisons of published data are often not feasible since earlier studies have not always specified the lobe of the rat prostatic complex used.

In contrast to oestradiol-17β, the hormone-cytotoxic agent, Estracyt, and its metabolite, estramustine were found, in the present study, to be much less effective in suppressing testosterone-induced DNA synthesis in cultured rat ventral prostate. Selective accumulation of ^{3}H-estramustine has been demonstrated in rat ventral prostate following treatment with either ^{3}H-estramustine phosphate (Plym-Forshell and Nilsson, 1974; Forsberg and Høisaeter, 1975; Høisaeter, 1976a, 1977) or ^{3}H-estramustine (Yamanaka et al., 1981a, 1981b; Symes and Milroy, 1982) and Yamanaka et al. (1981a, 1981b) have further shown that the ratio of radioactivity present in rat ventral prostate following the administration of labelled estramustine is significantly greater than that observed after treatment with

either oestradiol-17β or cyproterone acetate. While Høisaeter (1977) reported that after short-term incubation of minced rat ventral prostate with ^{3}H-estramustine, radioactivity was recovered in nuclear preparations, more recent studies indicate that subcellular localization of the estramustine complex is almost exclusively in the cytosol fraction (Yamanaka et al., 1981a, 1981b; Symes and Milroy, 1982) and the radioactivity associated with ^{3}H-estramustine is retained by the cytosol for at least 24 hours (Yamanaka et al., 1981a, 1981b). This suggests that a specific mechanism exists in the cytosol of rat ventral prostate for the retention of estramustine. Rat ventral prostate is known to contain a glycoprotein which constitutes almost 20% of the total cytosol protein and exhibits a high binding affinity for estramustine (Forsgren et al., 1979). This estramustine-binding protein is distinct from the classic androgen receptor (Yamanaka et al., 1981b; Høisaeter et al., 1981) but has several properties in common with previously described steroid binding proteins in the rat ventral prostate, which have been variously designated as "prostatic binding protein" (Heyns and DeMoor, 1977; Heyns et al., 1978a, 1978b; Heyns , 1980), "prostatein" (Lea et al., 1977) and "α-protein" (Liao et al., 1975, 1980). Thus, it appears that the presence of such a binding protein in rat ventral prostate may play a critical role in the retention of estramustine. A similar estramustine-binding protein has also been demonstrated in human prostatic tissue, but is present at a much lower concentration than in rat ventral prostate (Kirdani et al., 1981). Consequently, the high concentration of this receptor protein in rat ventral prostate may detract from the suitability of this tissue as a model for studying the effects of estramustine compounds. Furthermore, activation of hormone-cytotoxic compounds is dependent upon hydrolysis of the complex into its hormone and

cytotoxic parts within the target tissue (Mittelman, 1979; Nilsson and Muntzing, 1980; Pavone-Macaluso, 1982), yet Høisaeter (1975b, 1976a, 1977) was unable to demonstrate any hydrolysis of estramustine by rat ventral prostate *in vitro*. However, after "long-term" (3 days) Estracyt treatment *in vivo* evidence of small amounts of free oestrone were detected in rat ventral prostate (Høisaeter, 1976a, 1977). Recently, Symes and Milroy (1982) demonstrated that while the cleavage rate of estramustine in human prostatic tissue is dose-dependent, and is greater at 10^{-5}M than 10^{-8}M, the rate is nevertheless low. Thus, with regard to the present study, these results suggest that the inhibitory effect of the estramustine compounds at 4×10^{-5}M in the presence of 4×10^{-9}M testosterone may be due to the release of some oestradiol-17β from the hormone-cytotoxic complex. Furthermore, the present results have shown that the degree of inhibition in these cultures is comparable to that observed in cultures treated with 4×10^{-9}M oestradiol-17β and 4×10^{-9}M testosterone.

In contrast to the present study, Høisaeter (1975b) found that Estracyt (4×10^{-5}M) had a far more pronounced inhibitory effect than an equimolar concentration of oestradiol-17β on the incorporation of ^{3}H-TdR by rat ventral prostate following three days of organ culture in semi-defined (5% foetal calf serum) medium containing 4×10^{-6}M testosterone. However, it is of importance to note that treatment with 4×10^{-6}M testosterone alone did not stimulate any increase in ^{3}H-TdR uptake, which Høisaeter (1975b) attributed to a masking effect of endogenous hormones present in the serum supplement. Therefore, to evaluate the possible influence of serum, the present study investigated the effects of 4×10^{-5}M estramustine phosphate, estramustine and oestradiol-17β on the proliferative response to

4×10^{-6}M testosterone, both in the presence and absence of foetal calf serum. The results of this study have shown that in the presence of 5, 10 or 20% foetal calf serum, the estramustine compounds and oestradiol-17β were all equally inhibitory, whereas in the serum-free system only oestradiol-17β suppressed the testosterone-stimulated proliferative response. Thus, it appears that the presence of serum potentiates the antiandrogenic action of the estramustine compounds, while it reduces the inhibitory action of oestradiol-17β. These results were further substantiated by the marked degenerative changes observed in cultures treated with the estramustine compounds in serum-supplemented medium. However, cultures treated with these drugs in serum-free medium exhibited good maintenance, similar to that observed in the testosterone controls. In contrast, treatment with oestradiol-17β was markedly cytotoxic in serum-free cultures, but in the presence of serum it produced an antiandrogenic effect, characterized by flattened alveolar epithelium and loss of secretory activity. The presence of steroid hormone binding proteins, such as TEBG, in the serum supplement may be involved in the elimination of the cytotoxic effect of oestradiol-17β by reducing the concentration of free oestrogen in the medium. Similarly, the presence of steroid binding proteins may account for slight reductions observed in the stimulatory effect of testosterone on the proliferative activity of rat ventral prostate cultured in serum-supplemented medium. On the other hand, however, the results did not indicate that increasing the serum concentration from 5 to 10 or 20% further affected the action of either testosterone or oestradiol-17β.

Histologically, the results observed in the present study in serum-supplemented medium were remarkably similar to those of

Høisaeter (1975b), indicating that Estracyt had a more pronounced deleterious effect than oestradiol-17β on cultured rat ventral prostate. However, as Estracyt does not cause profound morphological changes in rat ventral prostate *in vivo* (Høisaeter, 1976b; Yamanaka *et al.*, 1977) or in serum-free organ culture, it appears that interactions between serum and Estracyt *in vitro* are involved in the production of the inhibitory response observed in serum-supplemented organ culture. Clearly, serum also influences the activity of oestradiol-17β *in vitro*. The ability of serum to modify the biological activity of hormones and chemotherapeutic agents *in vitro* further emphasises the complexity of serum supplements and may well represent a serious obstacle in the realization of the full potential of prostate organ culture as an experimental model for assessing the direct effects of hormones and drugs.

In contrast to organ culture, cell culture methodology involves disruption of the histological architecture of the tissue, thus identification of individual cell types can no longer be based on morphology alone, but requires the use of cell specific markers. The intense acid phosphatase activity of the human prostate epithelium is most frequently used as a histochemical marker for identifying isolated or cultured prostatic epithelial cells. Burstone (1958) first demonstrated the successful use of the azo-dye coupling technique for the histochemical localization of acid phosphatase activity. This technique involves incubation of the tissue specimen with a napthol AS-phosphate substrate and a diazonium salt. As a result of acid phosphatase activity, napthol AS is liberated, which immediately couples with the diazonium salt forming an insoluble and visible pigment at sites of acid phosphatase activity. More recently, Carson (1973) adapted this method

for differentiating between prostatic and non-prostatic acid phosphatase in cryostat sections of human tissue. This method is based on the fact that prostatic acid phosphatase is more resistant to inhibition by formalin than acid phosphatase from other human tissues. Cryostat sections of human prostatic tissue incubated in 10% neutral formalin for at least 24 hours remain acid phosphatase positive, whereas the enzyme activity in human liver and kidney is inactivated after 5 hours of formalin treatment (Carson, 1973; Stonington et al., 1975).

Results of the present study have also shown that human BPH remains acid phosphatase positive following 24 hours of formalin fixation, although the intensity of the reaction is reduced. In contrast to human prostatic tissue, however, the acid phosphatase reaction in young adult rat ventral prostate is less intense and disappears after 5 hours of formalin fixation. Similarly, the acid phosphatase activity in rat liver and kidney is no longer detectable after 5 hours of formalin treatment. Thus, the use of formalin resistance as a means of differentiating between prostatic and non-prostatic acid phosphatase does not appear to be directly applicable to rat tissue.

Differences in the intensity of the acid phosphatase reaction and the formalin sensitivity of human and rat prostatic tissue may be related to the absolute amount of acid phosphatase present in these tissues. Gutman and Gutman (1938a) reported that the amount of acid phosphatase in the human prostate is 1000 times greater than in any other human tissue and more recent studies have indicated that the ratio of acid phosphatase in human, as compared to rat, prostatic tissue is 1200:1 (Coffey and Issacs, 1980). Since human non-prostatic acid phosphatase activity disappears after 5 hours of formalin fixation, while

human prostatic acid phosphatase activity declines only after 24 hours of formalin treatment, Carson (1973) suggested that inactivation or destruction of the enzyme may be proportional to the duration of formalin fixation. Thus, the greater concentration of acid phosphatase in the human prostate, as compared with other human tissues or rat ventral prostate, may enable human prostatic tissue to remain acid phosphatase positive for prolonged periods of formalin treatment. Reductions observed in the intensity of the acid phosphatase reaction in human prostatic tissue following 24 hours of formalin fixation tend to support this hypothesis.

Unlike young adult rat ventral prostate, acid phosphatase activity in prostatic tissue from aged (> 12 months old) rats was not homogeneously distributed throughout the glandular epithelium, but rather it appeared in discrete clumps, which persisted after 5 hours of formalin fixation. A similar shift in the distribution of acid phosphatase activity in rat ventral prostate from both castrated and aged rats has been observed (Brandes, 1963, 1966, 1974b; Paris and Brandes, 1974; Mainwaring and Brandes, 1974; Brandes and Kirchheim, 1977) and attributed to the concentration of lysosomal enzymes in autophagic vacuoles associated with prostatic involution (Paris and Brandes, 1974). While the size and number of acid phosphatase positive granules appears to increase during androgen-deprivation or aging, suggesting increased acid phosphatase activity, it seems unlikely that synthesis of new enzymes occurs during regression of the tissue (Paris and Brandes, 1974). Alternatively, it has been suggested that lysosomal enzymes are degraded slowly, and possibly selectively retained by regressing prostatic tissue, thereby resulting in an apparent increase in acid phosphatase activity in atrophic prostatic epithelial cells (Paris et al.,

1972; Paris and Brandes, 1974). The increased formalin resistance of acid phosphatase observed in aged rat ventral prostate may, therefore, be related to the slow turnover of lysosomal enzymes during age-associated prostatic atrophy.

Although Rubenstein and Anderson (1980) indicated that the histochemical demonstration of formalin-insensitive acid phosphatase activity had been used to identify epithelial cells isolated from young adult rat ventral prostate, the cells were incubated in 10% neutral formalin for only one minute. However, the present study has shown that acid phosphatase activity in cryostat sections of young adult rat ventral prostate, liver and kidney fixed for at least fifteen minutes in 10% neutral formalin did not differ from that observed in replicate sections fixed either in cold acetone (15 minutes) or air dried without fixation. Moreover, prostatic epithelial cells derived from rat ventral prostate and fixed for one minute in formalin were indistinguishable from those which had been fixed in acetone (1 minute) or air dried without fixation. Hence, these results suggest that short-term incubations in formalin are not necessarily indicative of formalin-insensitive acid phosphatase activity in rat ventral prostate. However, as the acid phosphatase reaction in rat ventral prostate is confined to the epithelium, the histochemical demonstration of this enzyme still provides a means of distinguishing between epithelial and stromal cells derived from rat ventral prostate. Thus, in the present study, histochemically demonstrable acid phosphatase activity was used to identify epithelial cells isolated from mixed cell suspensions of young adult rat ventral prostate by isopycnic sedimentation.

Dow and Pretlow (1975) first investigated the use of density gradients as a means of separating purified populations of

epithelial cells from rodent prostate. However, using gradients ranging from 4.1 to 43% ($^{w}/w$) Ficoll^{R} (approximately 1.02 to 1.20g ml^{-1}), cells with positive acid phosphatase activity broadly overlapped with other prostatic cells bewteen densities of 1.05 to 1.12g ml^{-1}. On the other hand, both Dow and Pretlow (1975) and Rubenstein and Anderson (1980) found that purified fractions of viable epithelial cells could be successfully isolated from rodent prostate using gradients ranging from 2.7 to 5.5% ($^{w}/w$) Ficoll (approximately 1.01 to 1.10g ml^{-1}). However, preparation of the Ficoll gradient requires the use of special apparatus as the success of the separation is highly dependent upon the linearity and viscosity of the gradient, as well as on the speed and time of centrifugation (Pretlow and Boone, 1969; Pretlow, 1971; Helms et al., 1976; Rubenstein and Anderson, 1980). Alternatively, Pertoft et al. (1977) demonstrated the successful use of Percoll as a density gradient medium for separating viable cells of different types. Also, unlike Ficoll, the preparation of Percoll density gradients requires only a simple dilution procedure.

The present study, therefore, investigated the use of a discontinuous Percoll density gradient ranging from 1.02 to 1.10g ml^{-1} for the isolation of prostatic epithelial cells from young adult rat ventral prostate. Three reproducible cell bands were easily identified on the gradient: (1) a band containing cellular debris, (2) the epithelial cell enriched band and (3) a less distinct band containing primarily erythrocytes and some nucleated cells. The epithelial cell fraction, as identified by both cell morphology and positive acid phosphatase activity, sedimented at the 1.04g ml^{-1} interface on the discontinuous gradient. In contrast, Clark et al. (1982) reported that

epithelial cells derived from rat ventral prostate were recovered in the range of 1.05 to 1.065g ml^{-1} using a continuous Percoll density gradient. However, on the basis of cell morphology, it appears that the epithelial cell fractions described in both studies are equivalent. Furthermore, using a biochemical determination, Clark et al. (1982) demonstrated positive acid phosphatase activity in the epithelial cell fraction, while in the present study this was performed histochemically.

The successful use of the discontinuous Percoll gradient for isolating viable prostatic epithelial cells was further demonstrated by the exclusion of trypan blue dye and the ability of the isolated cells to rapidly establish monolayer cultures and incorporate ^{3}H-TdR. While the cells were apparently unable to proliferate in suspension cultures, as reflected by the decline in ^{125}I-UdR uptake, this is probably due to the lack of surface area available for attachment (Reid et al., 1980; Holley, 1980; Ham, 1981). As in the present study, however, previous investigations have also shown that epithelial cells isolated from rat ventral prostate generate monolayer cultures (Pretlow et al., 1977; Rubenstein and Anderson, 1980) which exhibit a high rate of ^{3}H-TdR incorporation after 72 hours in culture (Pretlow et al., 1977). The present procedure is, therefore, regarded as a rapid, reproducible and simple method for the isolation, characterization and culture of viable prostatic epithelial cells, which can be routinely performed in a standard laboratory.

CONCLUSIONS

Normal rat ventral prostate remains androgen-dependent and responsive in organ culture. This provides an *in vitro* system for investigating the direct effects of androgens, as well as antiandrogenic compounds, on prostatic growth, which can be evaluated both quantitatively and qualitatively using the ^{125}I-UdR labelling technique. However, variations in organ culture media and methodology can influence the proliferative activity of rat prostate *in vitro* and its response to hormone and drug treatment, thereby limiting the potential value of this experimental system.

In particular, the routine use of serum and, less frequently, insulin supplements in prostate organ culture can modify the proliferative response of the tissue. Serum supplements, which vary considerably as to species of origin and final concentration in the medium, often introduce unknown types and quantities of endogenous hormones. Furthermore, the presence of steroid-hormone binding proteins in most sera can alter the response to androgens by limiting the amount of free hormone available to the tissue. The presence of such steroid binding proteins can also necessitate the use of supraphysiological concentrations of testosterone in serum-supplemented organ culture, whereas equivalent concentrations in serum-free medium are often cytotoxic. Moreover, in chemically-defined organ culture, physiological concentrations of testosterone have been shown to effectively maintain the structural and functional integrity of rat ventral prostate in a state comparable to the intact gland. Similarly, serum may influence the effect of oestrogens on the proliferative response to testosterone and can also modify the actions of chemotherapeutic agents, such as Estracyt. Likewise, insulin modifies the proliferative activity by acting synergistically with testosterone and, possibly, serum. Although its mechanism

of action is unknown, insulin also appears to have a direct prostatic effect in chemically-defined organ culture. In view of the complexity of serum and insulin supplements and the fact that neither appear necessary for short-term organ culture of rat ventral prostate, their elimination from culture media should allow more precise definition of proliferative responses to hormone and/or drug treatment.

While the majority of studies employ the same basic organ culture technique, there is marked individual variability in experimental procedure which is often not adequately documented or fully justified. In chemically-defined organ cultures of young adult rat ventral prostate, the pattern of the proliferative response to stimulation with an optimal concentration of testosterone is characterized by a lag phase of approximately 48 hours, followed by a peak of DNA synthetic activity on day 4 and a decline thereafter, despite the continued presence of testosterone. However, variations in organ culture methodology and experimental procedure have been shown to modify the pattern and magnitude of this response. Moreover, the characteristic pattern of the response, itself, clearly demonstrates the difficulty in comparing results when different time points have been used to evaluate hormone or drug effects. Inherent differences in the various lobes of the rat prostatic complex may also be responsible for differences in the pattern of proliferative activity <u>in vitro</u>. While the ventral prostate is the most frequently studied lobe, widespread variation in the age of this tissue must be taken into consideration when attempting to compare proliferative responses. Furthermore, despite similarities between rat ventral and human prostate with regard to the mechanism of action of androgens, this lobe of the rat prostate is not necessarily the most representative

of the human prostate nor the most suitable for studying the action of estramustine compounds since it contains a high concentration of estramustine-binding protein. Thus, differences between the present results and other published data may be explained, at least in part, by variations in experimental conditions. In addition, histological criteria, the most common method used to assess the effects of hormones and drugs on prostatic tissue in vitro, renders comparisons difficult because of the inherent subjectivity of this technique. In contrast, quantitative organ culture, using the ^{125}I-UdR labelling technique, permits both DNA synthesis and the histological response to be evaluated in the same tissue sample. This advantage is of particular importance with regard to studies of the human prostate, where tissue is available only in limited quantities.

The emphasis placed on variations in culture media and methodology is not intended to discourage the use of organ culture but, rather, to stress the need for caution in the interpretation of results, particularly in comparing results between different experimental systems, unless the techniques are carefully co-ordinated. Clearly, organ culture technology has been refined to the point whereby the system can be used to obtain valuable insight into parameters governing the properties of prostatic tissue. Nevertheless, it is evident that further research and standardization is necessary before organ culture can be used with maximum efficacy within the intrinsic limitations of the system. Yet, despite a lack of uniformity in technique, extensive organ culture studies of rat ventral prostate have repeatedly shown that the effects of androgens on differentation and proliferation mimic that of the prostate in vivo. Thus, rat ventral prostate in

quantitative organ culture represents a particularly useful *in vitro* model for studying the direct actions of testosterone and its metabolites in a controlled and easily reproducible environment. This technique also allows investigation of interactions between other hormonal and non-hormonal factors which may influence the growth and/or function of prostate *in vivo*. Furthermore, the proliferative response of rat ventral prostate to testosterone stimulation in organ culture provides another approach for investigating the antiproliferative action of hormones and chemotherapeutic agents which may be of potential value in the treatment of prostatic cancer. In addition, the experience obtained from investigations of rat prostate in organ culture provides a valuable reference for the interpretation of hormonal and drug responses and a foundation for the further application of this technique to human prostatic tissue.

In contrast to the extensive investigations conducted on rat ventral prostate in organ culture, to date comparatively little has been achieved in prostate cell culture research, as techniques for the isolation of purified populations of viable prostatic epithelial cells have only recently become available. While the epithelial origin and the ability of these cells to proliferate *in vitro* has been demonstrated, a critical characterization of this experimental system must be undertaken before it can be used for studies of cell growth, differentiation and carcinogenesis. It is imperative that not only morphological characteristics of cells in culture be documented as epithelial and similar to their *in vivo* counterparts, but that they be shown to retain normal hormonal dependency. Nevertheless, *in vitro* model systems of prostatic epithelium will undoubtedly augment *in vivo* and organ culture studies of hormone responses of individual cell types. Moreover, this system appears

promising for studies on epithelial and stromal interactions in the prostate as well as for screening chemotherapeutic agents and investigating the role of cell aging in prostatic carcinogenesis. Thus, the use of both cell and organ culture models may provide concepts and techniques that will increase the understanding of the biology of normal, benign and malignant prostate cells.

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